DEPARTMENT OF THE ARMY
US ARMY INSTITUTE OF PUBLIC HEALTH
5158 BLACKHAWK ROAD
ABERDEEN PROVING GROUND MARYLAND 21010-5403

MCHB-IP-TEP

17 September 2013

MEMORANDUM FOR Environmental Acquisition and Logistics Sustainment Program (AMSRD-MSF/Ms. Kimberly A. Watts), U.S. Army Research Development and Engineering Command, 3072 Aberdeen Boulevard, Aberdeen Proving Ground, MD 21005

SUBJECT: Toxicology Study No. 85-XC-0ENTb-11, Protocol No. 0ENT-24-11-07-04, Acute Inhalation Toxicity and Blood Absorption of 3-Nitro-1,2,4-Triazol-5-One (NTO) in Rats, November – December 2011

1. An electronic copy of the subject report has been provided.

2. Please contact us if this report or any of our services did not meet your expectations.

3. The U.S. Army Public Health Command point of contact is Mr. Lee Crouse, Toxicology Portfolio, Toxicity Evaluation Program. He may be contacted at DSN 584-3980 or commercial 410-436-3980.

FOR THE DIRECTOR:

Encl

MARK S. JOHNSON
Director, Toxicology Portfolio

USAPHC
U.S. ARMY PUBLIC HEALTH COMMAND
5158 Blackhawk Road, Aberdeen Proving Ground, Maryland 21010-5403

Toxicology Study No. 85-XC-OENTb-11

Acute Inhalation Toxicity and Blood Absorption of 3-Nitro-1,2,4-Triazol-5-One (NTO) in Rats

Prepared by: Lee C.B. Crouse and Arthur J. O'Neill

Toxicology Portfolio
Toxicity Evaluation Program
Army Institute of Public Health

Specialty: 500C, Toxicity Study

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14. ABSTRACT

This toxicology study was conducted to determine the 4-hour inhalation median lethal concentration (LC50) of 3-Nitro-1,2,4 -Triazol-5-One (NTO) in male and female rats. Nose-only exposure to the highest-achievable aerosol atmosphere of NTO (0.18 mg/L) did not induce any compound-related mortality, adverse toxic signs, body weight changes, or gross necropsy findings. A secondary objective was to determine the effect that two different routes of administration (inhalation and oral) had on the absorption of the chemical into the bloodstream. Blood samples were collected and analyzed from exposed rats at 7 time points for those exposed via inhalation and 6 different time points for those given a calculated equivalent oral dose. The results of the blood absorption study indicated that, under the stated study conditions and limitations, acute exposure to NTO via inhalation appears to induce higher whole blood concentrations in laboratory rats compared to those exposed via oral gavage.

15. SUBJECT TERMS

3-Nitro-1,2,4-Triazol-5-One, NTO, acute inhalation toxicity, LC50, rat, oral gavage

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Study Title

Toxicology Study No. 85-XC-0ENTb-11
Protocol No. 0ENT-24-11-07-04
Acute Inhalation Toxicity and Blood Absorption
of 3-Nitro-1,2,4-Triazol-5-One (NTO) in Rats

Data Requirement

None

Authors

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Study Completed On

July 2013

Performing Laboratory

U.S. Army Public Health Command
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5158 Blackhawk Road
Aberdeen Proving Ground, MD 21010-5403

Laboratory Project ID

Protocol No. 0ENT-24-11-07-04

STATEMENT OF DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this report on the basis of its falling within the scope of TSCA § 790.7 (a) – (d).

Company:_____

Company Agent
Typed Name:_____ Date:_____

Title:_____ Signature:_____

Toxicology Report No. 85-XC-0ENTb-11, Nov – Dec 2011

Submitted by: Army Institute of Public Health
Toxicology Portfolio
MCHB-IP-TEP
5158 Blackhawk Road
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Director, Toxicology Portfolio

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Date

Good Laboratory Practice Compliance Statement

The study described in this report was conducted in compliance with Title 40, Code of Federal Regulations (CFR), Part 792, Good Laboratory Practice Standards, except for the following:

1. The concentrations of the test article dosing suspensions for the multi-time point blood absorption portion of the study were not verified analytically in accordance with Good Laboratory Practice Standards. The accuracy of the data reported is considered sufficient for the purposes of the acute study.

2. The statistical analyses of the multi-time point blood absorption data were conducted by the U.S. Army Public Health Command statisticians. It is not known if these analyses were conducted in accordance with Good Laboratory Practice Standards.

3. The animal room relative humidity was below the targeted range of 30-70% for a period of approximately 28 hours during the acclimation period for the pilot study animals.

ARTHUR J. O'NEILL
Study Director
Toxicity Evaluation Program

13 SEPT 2013
Date

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TOXICOLOGY STUDY NO. 85-XC-0ENTb-11
PROTOCOL NO. 0ENT-24-11-07-04
ACUTE INHALATION TOXICITY AND BLOOD ABSORPTION OF 3-NITRO-1,2,4-TRIAZOL-5-ONE (NTO) IN RATS
NOVEMBER – DECEMBER 2011

1 Summary

1.1 Purpose

This study was conducted to determine the 4-hour, inhalation median lethal concentration (LC_{50}) of 3-Nitro-1,2,4-Triazol-5-One (NTO) in male and female rats. The LC_{50} is defined as the calculated atmospheric concentration of test substance expected to cause the death of 50 percent of exposed animals either on the day of exposure or within at least 14 days post exposure. In the event that no deaths occur among rats exposed to the highest-obtainable concentration, the LC_{50} is considered greater than the given concentration and no further testing is required. A secondary objective was to determine the effect that two different routes of administration (inhalation and oral) had on the absorption of the chemical into the bloodstream. Blood samples were collected from exposed rats at seven different time points for rats exposed via inhalation and at six different time points for rats exposed via oral gavage to measure the absorption of NTO into the blood and determine the degree of effect that the exposure route had on the absorption of NTO into the blood. In addition to blood absorption, rats were also evaluated for body weight changes, and clinical observations.

1.2 Conclusions

Rats were exposed nose-only to a 0.18 milligram per liter (mg/L) aerosol atmosphere of NTO for a single 4-hour exposure. No test compound-related mortalities occurred in rats exposed during the study and no adverse toxic signs, body weight changes, or gross necropsy findings were observed in exposed rats. The results of the LC_{50} portion of this study indicate that acute inhalation exposure to the highest-achievable concentration of NTO aerosol (0.18 mg/L) is relatively nontoxic to rats.

The results of the multi-time point blood absorption portion of the study indicated that, under the stated study conditions and limitations, acute exposure to NTO via inhalation appears to induce higher whole blood concentrations in laboratory rats compared to those exposed via oral gavage.

2 References

See Appendix A for a list of references.

3 Authority

MIPR No. MIPR1JDATHR142. This toxicology study addresses, in part, the environmental safety and occupational health requirements outlined in Army Regulations (AR) 200-1, AR 40-5, and AR 70-1; Department of Defense Instruction 4715.4; and Army Environmental Requirements and Technology Assessments (Department of the Army (DA), 2007a and b; DA, 2003; Department of Defense (DOD), 1996; and U.S. Army Environmental Command (USAEC), 2009). It was performed as part of an on-going effort by the U.S. Army Environmental Quality Technology (EQT), Ordnance Environmental Program Pollution Prevention Team, to produce safer ordnance. This program is under the

direction of the U.S. Army Research, Development, and Engineering Command Environmental Acquisition Logistics & Sustainment Program and EQT Pollution Prevention.

4 Background

As a result of the DOD-wide initiative to improve munitions safety, the U.S. Army is developing insensitive munitions (IM) for incorporation into its inventory of conventional ammunition and missiles. The Army's IM Program is dedicated to developing munitions that reliably perform as they are intended but are less prone to inadvertent initiation from external stimuli such as bullet/fragment impact, heat from fire, and shock from neighboring explosions (Duncan, 2002). The production of insensitive munitions requires the use of intrinsically insensitive explosives that contribute to lower order responses to inadvertent external stimuli. Despite the slightly lower performance of NTO compared to TNT, there has been a renewed interest in its use in explosive formulations based on the lower sensitivity as a melt-cast medium observed during testing and the less stringent shipping requirements. This has lead to the development of a range of melt-castable explosives at Picatinny Arsenal, collectively known as "PAX" explosives (Davies and Provatas, 2006). To support the possible fielding and full-scale production of these PAX explosives, occupational exposure guidelines need to be developed and refined using toxicity data in a mammalian system to assess any occupational health hazards associated with the use and production of this material.

NTO is being investigated as a less sensitive direct replacement for traditional explosives such as TNT and RDX. NTO is a crystalline powder that is one of the components used in the formulation of an insensitive explosive referred to as IMX101. NTO was first reported in 1905 but was not used as an explosive until the early 1980's when it was discovered that the French were developing a "new insensitive explosive", which was later reported to be NTO. Renewed interest in the energetic properties of NTO has been fueled by the need to develop munitions that are less prone to inadvertent initiation during transport and routine handling. The reduced sensitivity to environmental stimuli and nearly equal performance during testing make NTO-based formulations desirable replacements for currently fielded munitions (Spear et al, 1989; Smith and Cliff, 1999). In addition to minimizing collateral damage from weapon or ordnance accidents, insensitive munitions offer logistical advantages on the battlefield. As modern battlefields increasingly shift into populated urban centers, insensitive munitions inventories represent a less desirable target for terrorists and minimize the threat to surrounding communities. Less sensitive munitions could potentially be more cost effective and efficient to transport if granted reduced DOD/Department of Transportation hazard classification rankings (DA, Rapid Action Revision (RAR) 2009).

A literature search was conducted prior to the initiation of the study revealing a limited amount of preliminary toxicity data. The reported mouse and rat oral lethal dose (LD_{50}) values were both greater than 5000 milligrams per kilogram (mg/kg). In addition, NTO was reported to be a mild skin and eye irritant but was not a dermal sensitizer (Los Alamos National Laboratory, 1985). A subchronic oral toxicity study in rats on NTO was performed by this Command in 2008. The subchronic study on NTO revealed significant reductions in both testes and epididymides weights and weight ratios at dosages of 315 mg/kg-day and above. Significant reductions in sperm counts were also noted at dosages of 315 mg/kg-day and above. Histopathology performed on the 90-day tissues revealed significant incidences of testicular hypoplasia at dosages of 315 mg/kg-day and above as well as insignificant, less severe, testicular hypoplasia at dosages of 100 mg/kg-day and below (U.S. Army Public Health Command (USAPHC) (Provisional), 2010a). A repeated-dose, 14-day range finding study was also completed prior to the initiation of the 90-day study. In this 14-day study, testes weights and weight ratios were significantly reduced compared to controls in male rats administered 500 mg/kg-day NTO and above. No significance was observed for epididymus

weights or weight ratios compared to controls and histopathology was not performed on any tissues from the 14-day study. The data from the oral subchronic study was used to calculate an occupational exposure level (OEL) for NTO. In addition to establishing an LC_{50} for NTO, the data from this study will also be used to refine the established OEL that was previously extrapolated from oral toxicity data. The following table identifies the critical dates of this study.

Table 1. Critical Study Events

Critical Event	Date of Event
Animal Use Protocol Approved	July 29, 2011
Animals Received for Pilot Exposure	November 2, 2011
Pilot Exposure Conducted	November 7, 2011
Pilot Exposure Necropsy	November 10, 2011
Animals Received for LC_{50} Study	November 16, 2011
LC_{50} Study Conducted	November 21, 2011
LC_{50} Necropsy	December 5, 2011
Animals Received for Blood Absorption Study	November 30, 2011
Inhalation and Gavage Exposures for Blood Absorption Study	December 1 and 2, 2011
Experimental Completion	December 5, 2011
Study Completion	July 2013

5 Materials

5.1 Test Substance

NTO is a light green to white crystalline solid with no odor. The chemical formula is $C_2H_2N_40_3$ and the molecular weight is 130.06 grams per mole. The manufacturer has stated that NTO has a melting point of 268-271°C and a specific gravity of 1.93 (BAE Systems, 2009). The material was supplied by Ordnance Systems, Inc., Kingsport, Tennessee and identified as lot number BAE 07B 305001. The compound purity was performed by the manufacturer and reported as 99.6% pure by HPLC analysis. The material was shipped to the U.S. Army Research, Development Engineering Command, Engineering Directorate, Pyrotechnics Team, APG-EA, MD 21010. For each dosing suspension and inhalation solution, the calculated amount of NTO was weighed and placed in a ceramic mortar. The NTO was then wetted with a measured amount of distilled water and ground with a mortar and pestle to a fine consistency. The slurry was transferred to a volumetric flask and the mortar was rinsed with a measured amount of distilled water to remove any remaining slurry. The remaining distilled water was then added to the suspension to achieve the calculated concentration. Due to the large volume of test solution required for the inhalation phases of this study, each batch was warmed slightly on the low setting of a hot plate while being mixed to facilitate the formation of a solution. Once the NTO went into solution, it would typically remain in solution at room temperature for a period of approximately 7 days.

5.2 Animals[*†]

[*] Research was conducted in compliance with DoD and federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. National Academy Press, Washington, D.C. 1996.

Each phase of this study was conducted using young adult male and female Sprague-Dawley rats obtained from Charles River Laboratories, Wilmington, Massachusetts. A total of two rats were received for the acute inhalation pilot test. A total of 10 rats were received for the acute inhalation (LC_{50}) test. Six of the 10 rats received were assigned to the test group while the remaining rats were used for the range finding blood absorption test. A total of 14 rats, each with a subcutaneous femoral artery catheter in place, were ordered for the multi-time point blood absorption test. Twelve of the 14 rats received were assigned to either inhalation or oral gavage test groups (one additional rat of each sex was ordered to ensure that a sufficient number of rats were available for testing in case there were clogging issues with any of the catheters following shipment). At the time of their arrival, the animals were approximately 7 weeks old with the exception of the catheterized animals which were 8 weeks old. The attending veterinarian examined the animals and found them to be in acceptable health. Due to potential clogging issues, rats received with femoral artery catheters did not have the standard 5-day acclimatization period, and therefore, were used for testing 1-2 days following their arrival to the testing facility. All rats were maintained in a temperature-, relative humidity-, and light-controlled room. The animal room environmental conditions were maintained at 69.4 ± 0.76°F, and 49.6 ± 7.15% relative humidity with a 12-hour light/dark cycle. A certified pesticide-free rodent chow (Harlan Teklad®, 8728C Certified Rodent Diet) and drinking quality water were available *ad libitum* except during the 4-hour exposure period. Rats were housed singly in 17-inch (length) x 9-inch (width) x 8-inch (height) solid bottom polycarbonate boxes with ALPHA-dri® bedding and suspended on a cage rack equipped with an automatic water-nipple system. Each rat was uniquely identified by number using cage cards. In addition, an animal identification number was recorded on the tail of each rat with a water-insoluble marker prior to exposure so that individual rats could be identified after exposure. (Teklad® Certified Rat Diet is a registered trademark of Harlan, Teklad. ALPHA-dri® is a registered trademark with Shepherd Specialty Papers.)

5.3 Quality Assurance

The USAPHC Quality Systems Office audited critical phases of these studies. Appendix B provides the dates of these audits along with the audited phase.

5.4 Study Personnel

Appendix C contains the names of persons contributing to the performance of these studies.

6 Methods

6.1 General Description

This entire study protocol consisted of four separate tests utilizing two different methods of compound administration. Two of the tests served as pilot or range finding tests for the more definitive acute LC_{50} and multi-time point blood absorption tests. The acute inhalation pilot test, using only one rat per sex, was conducted to verify if the aerosol generation method was feasible for animal exposures and to determine if the relatively low pH (~2.3) of the NTO/distilled water solution was going to cause additional unwanted health effects in exposed animals beyond the inherent toxicity caused by NTO. The range finding blood absorption test, utilizing two additional rats per sex ordered for the acute inhalation LC_{50} test, was conducted to determine if the inhalation

† The studies reported herein were performed in animal facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

and oral exposure levels anticipated for the multi-time point blood absorption test would allow for an accurate determination of the NTO concentration in rat blood. The results of the two pilot or range-finding tests do not contribute meaningfully to the primary objectives of this study and the results will not be reported herein.

For the acute inhalation (LC_{50}) test, a single group of three male and three female rats were exposed to an atmospheric concentration of NTO aerosolized in air for a period of 4 hours. Rats were observed for mortality and clinical signs of toxicity during exposure and immediately following exposure. During a 14-day post-exposure recovery period, rats were observed each day for mortality and clinical signs of toxicity and weighed on selected post-exposure days. All rats were euthanized by carbon dioxide asphyxiation following the recovery period and underwent a gross pathological examination.

The multi-time point blood absorption test involved the use of two groups of three male and three female rats implanted with femoral artery catheters. One group of six rats was exposed nose-only to an NTO aerosol for 3.8 – 4 hours. The second group of six rats was administered a single oral dose via gavage at calculated equivalent doses to those exposed via inhalation. The absorption of NTO into the bloodstream of the exposed rats via inhalation was determined from blood samples collected from each surviving rat at seven nominal time points during the exposure: (1) approximately 1-2 hours prior to the exposure (2) approximately 1 hour after the initiation of the exposure period, (3) approximately 2 hours after the initiation of the exposure period, (4) at the conclusion of the 4-hour exposure period, (5) approximately 4 hours after the conclusion of the exposure period, (6) approximately 8 hours after the conclusion of the exposure period and (7) following an overnight recovery period. The absorption of NTO into the bloodstream of the exposed rats via oral gavage was determined from blood samples collected from each surviving rat at six nominal time points: (1) approximately 1-2 hours prior to dosing (2) approximately 1 hour after dosing, (3) approximately 2 hours after dosing, (4) approximately 4 hours after dosing, (5) approximately 8 hours after dosing, (6) following an overnight recovery period. In addition to blood analysis, rats were also placed in metabolism cages shortly after the last blood sample on the day of exposure (during overnight recovery) for collection of urine samples. Clinical observations were collected on all exposed rats throughout the day of exposure/dosing and prior to euthanasia following the collection of the final blood sample.

The experimental design and general procedures related to the exposure chamber generation and the oral gavage procedures of this study were conducted under the USAPHC Portfolio of Toxicology Standing Operating Procedure (PTOX SOP) for conducting acute inhalation and acute oral toxicity studies (USAPHC, 2010a and 2010b). These SOPs are modeled on the U.S. Environmental Protection Agency (EPA) Office of Chemical Safety and Pollution Prevention (OCSPP) Health Effects Test Guidelines, OCSPP 870.1300, Acute Inhalation Toxicity and OCSPP 870.1100, Acute Oral Toxicity (EPA, 1998 and 2002). By guideline, acute inhalation studies are typically conducted using at least 5 rats/sex, however, these inhalation exposures were conducted with 3 rats/sex. The flexible barriers used to prevent NTO aerosol from escaping around the head of each animal required the use of a chamber faceplate that could only accommodate 3 rats/sex/exposure.

6.2 Inhalation Exposures for Acute LC_{50} Test and Inhalation Portion of Multi-Time Point Blood Absorption Test

6.2.1 Selection of Exposure Chamber Design Concentration

The design concentration of the NTO aerosol atmosphere in the exposure chamber was selected based on the highest obtainable concentration achieved, up to a limit concentration of 2000 milligrams per cubic meter (mg/m^3), during method development work. Multiple attempts were made during method development to generate a limit concentration of 2000 mg/m^3, however, due to the limited solubility of NTO in distilled water as well as the inherent inefficiency of aerosol atmospheres, the highest reproducible NTO atmosphere concentration obtained was approximately 200 mg/m^3. The NTO could not be generated as a dry particulate atmosphere because of its' energetic properties and large particle size. The use of larger-bore nebulizers, resulting in higher feed rates, caused the particle size of the test atmosphere to be primarily outside of the respirable range (1-4 microns) for rats. Therefore, the design concentration for the current study was selected to be approximately 200 mg/m^3.

6.2.2 Exposure System

6.2.2.1 Test Atmosphere Generation

Chamber atmospheres of NTO aerosol were generated dynamically in air within the exposure chamber. Test atmospheres were generated by suspending the aerosolized test material in air. A 17 milligram per milliliter (mg/mL) NTO/distilled water solution was siphoned into dual 1650 liquid cap Spraying System nebulizers equipped with #64 air caps. Filtered houseline air, metered with glass tube rotometers, was passed through one port of each nebulizer at a rate of approximately 15 liters per minute for each nebulizer. The other port of each nebulizer was connected to a ¼ inch diameter section of Teflon® tubing which was placed in the NTO solution. The liquid feed rate of each nebulizer was controlled by the volume of houseline air being metered through each nebulizer (see Figure 1). Each nebulizer was siphoning approximately 22 milliliters/minute of NTO solution at 15 liters/minute of air. Test atmospheres were first exhausted through an impinger containing water to scrub the NTO and then through an impinger containing desiccant to remove moisture using a Radeco model AVS-28A vacuum pump prior to discharge into a fume hood. The rate of exhaust from the exposure chamber was sufficient to minimize leakage of the test atmosphere from the chamber. (Teflon® is a trademark of E.I. DuPont de Nemours, Inc.)

6.2.2.2 Exposure Chamber

The exposure chamber was a glass cylinder (12-inch height x 16-inch diameter x ¼-inch walls) with a nominal internal volume of approximately 36 liters. The open end of the exposure chamber was fitted with a polymethyl-methacrylate faceplate. The faceplate functioned to seal the opening of the exposure chamber and to support the nose-only restrainers during the exposure.

6.2.2.3 Exposure Mode

Animals were exposed nose-only to aerosolized atmospheres of NTO such that only their heads were positioned within the exposure chamber and the bodies of these rats were positioned outside the exposure chamber. The area of the exposure cylinder between the head and body of the rats was separated by a flexible neoprene barrier in an attempt to minimize leakage of the test atmosphere from the exposure chamber. Rats were individually contained during each exposure in perforated, stainless steel cylinders with conical nose pieces. Rats were positioned in the exposure cylinder such that their noses were at the conical end of the cylinder. In order to secure the rat in this position, a plastic disk with a hole in the center was inserted over the tail of each rat and positioned within the cylinder close to the base of the rat's tail so as to prevent the rat from backing out of the rear of the cylinder. Each exposure cylinder was inserted into one of the holes in the

faceplate of the exposure chamber such that the head of each rat extended into the exposure chamber (nose-only).

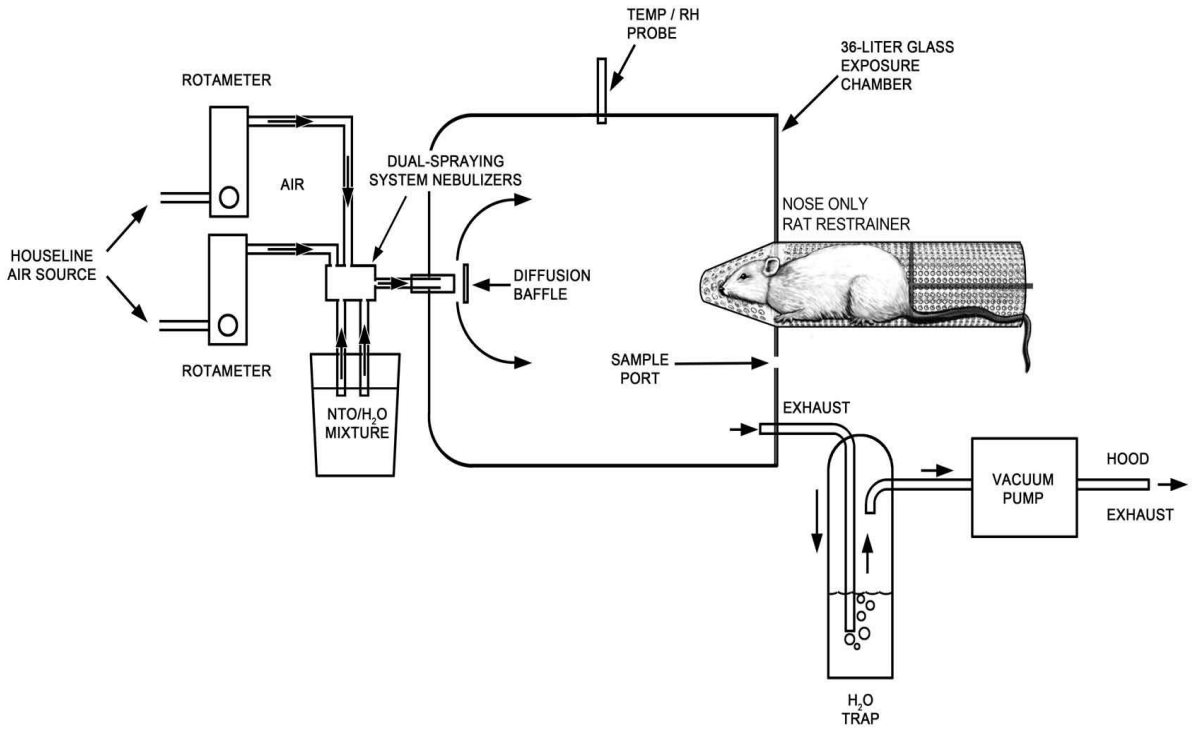

Figure 1. Generation/Exhaust/Exposure System

6.2.2.4 Exposure Duration

All rats used for the acute LC_{50} test and the inhalation portion of the multi-time point blood absorption test were exposed for 4 hours to the test atmosphere. However, one female rat exposed during the blood absorption test was removed approximately 12 minutes early due to signs of apparent hypothermia induced by the aerosol test atmosphere. In order to accommodate the blood collection schedule for animals in the multi-time point blood absorption study, these rats were loaded into the exposure system chamber in staggered increments. The time that each rat was loaded into the exposure system was recorded in the study records and represented the beginning of the exposure period for that rat. These rats were then removed from the exposure chamber for 6-9 minute periods for the 1- and 2-hour bleed time points. The total time that the rats were not in the exposure chamber during the 1-and 2-hour bleed time points was made up at the end of the exposure to ensure that each rat received a 4-hour exposure. In addition, to insure that each animal was exposed to the same NTO concentration regardless of their location in the chamber faceplate, all of the rats were systematically rotated throughout the positional ports in the faceplate during both inhalation exposures. For the acute LC_{50} test the animals were rotated

halfway through the exposure and for the multi-time point blood absorption test the animals were rotated every hour during the 4-hour exposure.

6.2.3 Characterization of Exposure Chamber Atmosphere

6.2.3.1 Test Substance Atmospheric Concentration

The atmospheric concentration of NTO aerosol was determined at regular intervals (e.g., 30-minutes) during both inhalation exposures. A total of 9 to 11 samples were collected from the exposure chamber during each of the two exposures. Known volumes of chamber atmosphere were drawn from a sampling port in the exposure chamber faceplate representative of the animals' breathing zone. Samples were drawn through a 25 millimeter (mm) filter cassette that contained a dried, pre-weighed Gelman glass fiber (Type A/E) filter. All filters were weighed on a Cahn model C-34 Microbalance. Once each sample was collected and a wet post-sample weight was determined, the filters were placed in a desiccator overnight to allow the distilled water to evaporate. The following day, a dry post-sample weight was determined which represented the concentration of NTO in the test atmosphere. The atmospheric concentration of NTO was calculated from the difference in the pre- and post-sampling filter weights divided by the volume of chamber atmosphere sampled.

6.2.3.2 Particle Size Analysis

Two samples to determine atmospheric particle size distribution (mass median aerodynamic diameter) of the NTO test atmosphere were collected during the acute LC_{50} exposure. One sample was collected during the first hour of the exposure and the second sample was collected during the final hour of the exposure. A single particle size sample was collected during the final hour of the exposure for the inhalation portion of the multi-time point blood absorption study. All particle size samples were collected using a Sierra® Series 210 8-Stage Cascade Impactor fitted with a Cyclone Preseparator and Anderson model SE113 Constant Flow Air Sampler. Particle size sample data were analyzed by log normal regression of particle size versus cumulative relative mass (USAPHC, 2010d). (Sierra® is a trademark of Sierra Instruments Inc.).

6.2.3.3 Environmental Monitoring

Chamber airflow was set at the beginning of the exposure to achieve at least 10 air changes per hour. The airflow fed through each nebulizer was monitored continually with 2 Fischer & Porter model 10A1338 flowmeters. Chamber temperature was targeted at 22 ± 2 ºC and relative humidity was targeted between 30 and 70 percent. Chamber temperature and humidity were measured with an Omega model RH411 Digital Thermo-Hygrometer thermometer. Airflow, temperature, and relative humidity readings were recorded multiple times during each exposure.

6.3 Oral Gavage Portion of Multi-Time Point Blood Absorption Test

6.3.1 Calculation of Equivalent Oral Dose

The purpose of the multi-time point blood absorption test was to compare the absorption of NTO into the blood of rats exposed via inhalation to that of rats given an equivalent oral dose. On the day following the conclusion of the inhalation portion of the test, an average chamber concentration was calculated for the 4-hour exposure using the dried gravimetric filter samples collected during

the exposure. The following formula was employed to estimate an equivalent oral dose for the animals to be gavaged (Rusch, 2009):

$$\text{Oral Dose (mg/kg)} = \frac{\alpha * \text{exposure level} * \text{minute volume} * \text{exposure length}}{\text{body weight}}$$

where:
α = amount retained in respiratory system (assume 0.9 or 90% in the absence of additional information)
exposure level = average concentration of inhalation exposure (mg/m^3)
minute volume = amount of air inhaled per minute (approx. 0.00016 m^3/min for rats)
exposure length = total minutes of inhalation exposure
body weight = animal weight (kg)

All calculations were performed using body weights obtained within an hour of the dosing procedure.

6.3.2 Administration of Test Substance

The animals were not fasted prior to dosing since the inhalation animals were not fasted prior to exposure. The dosing procedure for each animal was staggered by a period of approximately 4 minutes to accommodate the blood sampling schedule and all dosing was performed using a 16 gauge x 2-inch stainless steel gavage needle. The same 17 mg/mL stock solution of NTO in distilled water that was used for the inhalation exposures was used for the oral gavage and dosage volumes ranged from 1.42 – 2.25 mL of solution per kilogram bodyweight.

6.4 Blood/Urine Sample Analysis

6.4.1 Collection of Blood Samples

The concentration of NTO absorbed into the bloodstream of exposed rats via nose-only inhalation or oral gavage was determined from blood samples collected from each surviving rat at seven selected time points for the inhalation portion and six selected time points for the oral gavage portion. All blood samples were preserved at the time of collection by immediately injecting the 100 microliter aliquot of the blood sample into 1 mL of a 25/75 mixture of water and acetonitrile. Samples were tightly capped and refrigerated at approximately 4°C immediately after collection until the time of delivery for analysis.

6.4.2 Collection of Urine Samples

Surviving animals exposed via inhalation or oral gavage were placed in metabolism cages following the 8-hour post-exposure blood sample on the day of exposure for overnight urine collection. Each animal was in the metabolism cage for a period of approximately 14-15 hours. The following day the animals were removed from the metabolism cages for the final blood sample and the urine samples were transferred to Becton-Dickenson Falcon™ 15-mL conical centrifuge tubes. Each sample was centrifuged for approximately 30-minutes at slow speed to remove any particulate contamination. The liquid urine samples were then transferred to new centrifuge tubes and frozen at approximately -35°C until the time of delivery for analysis. (Falcon™ is a trademark of Becton, Dickinson and Company.)

6.4.3 Analysis of Blood/Urine Samples

Army Institute of Public Health (AIPH) Laboratory Science (LS) personnel analyzed the concentration of NTO in the blood and urine samples. Prior to LS analysis, the urine samples were thawed and diluted 10x in a 25/75 mixture of deionized water and acetonitrile. The blood samples were centrifuged to separate the blood cells from the collection medium. Both the blood and urine samples were diluted as necessary to bring the sample concentrations within the range of instrument calibration. All blood samples were analyzed using an Agilent 6100 LC/MSD with a Thermo Scientific Hypercarb 150 X 4.6mm, 5 micron (µm) particle size analytical column. The urine samples were analyzed using a DIONEX U3000 LC-UV with a Thermo Scientific Hypercarb 150 X 4.6mm, 5µm particle size analytical column. Confirmation of the NTO was then performed by LC-MS.

6.5 Body Weights and Clinical Observations

All rats were weighed and observed prior to all exposures. The animals used for the acute LC_{50} test were weighed and observed on the day of exposure, the day following exposure, several times during the 14-day observation period, and just prior to euthanasia. The animals used for the multi-time point blood absorption test were weighed and observed on the day of exposure and observed only on the day following exposure prior to euthanasia.

6.6 Pathology

All rats exposed for the acute LC_{50} test were euthanized by carbon dioxide asphyxiation following the 14-day observation period and underwent a gross pathological examination. Animals used for the multi-time point blood absorption test were euthanized by a sodium pentobarbital injection into the femoral artery catheter following the final blood sample. The rat exposed as part of the acute LC_{50} test that died during exposure underwent a gross pathological examination shortly after death.

6.7 Statistical Analysis of Data

For the acute LC_{50} toxicity test, the test substance generated at the highest-obtainable concentration did not result in any test substance-related mortality. Therefore, the LC_{50} for NTO generated as described above is reported as greater than the highest-obtainable concentration. For the multi-time point blood absorption test, a comparison of the concentration of NTO in the blood samples across times was performed with an analysis of variance (ANOVA) on the area under curve (AUC) for each rat. The AUC for each rat was calculated using the rectangle method. An ANOVA was then performed using both gender and route of administration as factors. Statistical significance for all tests was defined as $p<.05$. Descriptive statistics (e.g., mean, standard deviation, and standard error of the mean) were used to summarize experimental data (e.g., atmospheric concentrations).

7 Results

7.1 Generation Method Development

Prior to the initiation of the inhalation test exposures, pre-test trials were conducted to determine the most suitable method of generating aerosol atmospheres of NTO. The initial goal of this preliminary work was to determine if a reasonably stable atmospheric concentration of approximately 2 mg/L NTO could be achieved. The generation system used for the animal exposures was selected based on the energetic nature of NTO, the solubility of NTO in water, and

the ability to generate stable atmospheres of NTO at the highest-achievable concentration. The same generation system was used for both inhalation phases of the study.

7.2 Chamber Distribution of Test Atmosphere

Prior to initiation of the inhalation test exposures, a study of the chamber distribution of the NTO aerosol concentration was performed (USAPHC, 2010c). Significant differences between gravimetric samples taken from the sample port on the side of the chamber and those obtained from the chamber faceplate were observed during method development exposures. The distribution of the NTO aerosol atmosphere at various locations in the chamber faceplate was then gravimetrically determined during a pretest trial. Significant differences between the concentrations observed in the middle of the faceplate and those obtained from the top and bottom of the faceplate were observed. Therefore, all gravimetric exposure chamber samples as well as animal exposures were performed from ports in the middle of the faceplate. In addition, to insure that each animal was exposed to the same NTO concentration regardless of their location in the chamber faceplate, the animals were rotated during both inhalation exposures. For the acute LC_{50} test the animals were rotated halfway through the exposure and for the multi-time point blood absorption test the animals were rotated every hour during the 4-hour exposure. Distribution of atmospheric NTO concentrations in the exposure chamber data are presented in Appendix D.

7.3 Exposure Chamber Concentration and Conditions During Animal Exposure

7.3.1 Atmospheric Concentration of Test Chemical

One inhalation exposure was conducted for the acute LC_{50} test and one inhalation exposure was conducted for the multi-time point blood absorption test. The mean atmospheric concentration of NTO aerosol in the exposure chamber during the acute LC_{50} test was determined to be 0.18 ± 0.02 mg/L. The mean atmospheric concentration of NTO aerosol in the exposure chamber during the multi-time point blood absorption exposure was determined to be 0.21 ± 0.03 mg/L. Exposure concentration data are presented in Appendix E and summarized in Table 2.

Table 2. Summary of NTO Exposure Chamber Concentrations

Exposure	Atmospheric Concentration of NTO (mg/L)			
	Mean	S.D.	Range	N
LC_{50}	0.18	0.02	0.16-0.22	9
Multi-Time Point Blood Absorption	0.21	0.03	0.18-0.28	11

Legend:
mg/L = milligrams per liter
S.D. = standard deviation
N = number of samples collected
Note:
Values reported to 2 significant figures

7.3.2 Nominal Concentration of Test Substance

The nominal concentration is the theoretical atmospheric concentration calculated when the total volume of test substance delivered to the generation system, taking into account the density of the test chemical, is divided by the total airflow of the generation system. The nominal concentration of the total NTO/distilled water test atmosphere for both exposures was calculated to be 1481 mg/L ([44 mL/min x 1.01 g/mL (density) x 10^3 (mg/g)] ÷ 30 liters/min). The nominal concentration of the NTO component of the test atmosphere for both inhalation exposures was calculated to be 24.9 mg/L ([44 mL/min x 17 mg/mL] ÷ 30 liters/min). Aerosol test atmospheres are typically one of the least efficient atmospheres that can be generated due to wall-loss of the test material on the sides of the chamber and settling of the aerosol particles. Multiple attempts were made during method development to efficiently generate NTO at concentrations closer to the calculated nominal concentrations, however gravimetric samples obtained from the exposure chamber consistently averaged 0.2 mg/L.

7.3.3 Particle Size of Test Substance

The particle size of the test atmosphere, characterized by measurement of the Mass Median Aerodynamic Diameter (MMAD), was determined twice during the conduct of the acute LC_{50} test and once during the conduct of the inhalation portion of the multi-time point blood absorption test. The MMAD of the LC_{50} test atmosphere ranged from 4.6 to 5.0 µm, with 0.5-1.3 percent of the particles less than 1 µm, 19-26 percent of the particles less than 4 µm, and 87-88 percent of the particles less than 10 µm. The MMAD of the test atmosphere for the inhalation portion of the multi-time point blood absorption study was 4.8 µm, with 0.9 percent of the particles less than 1 µm, 24 percent of the particles less than 4 µm, and 87 percent of the particles less than 10 µm. Particle size data is summarized in Table 3.

Table 3. Summary of NTO Exposure Particle Sizes

Exposure	Mass Median Aerodynamic Diameter (µm)	Geometric Standard Deviation	% Particles by Mass		
			<1 µm	<4 µm	<10 µm
LC_{50}*	4.6 – 5.0	1.8 – 1.9	0.5 - 1.3	19 - 26	87 - 88
Multi-Time Point Blood Absorption	4.8	1.8	0.90	24	87

Legend:
µm = micrometer
% = percent
Note:
*Two particle size samples collected during exposure.

7.3.4 Exposure Chamber Environmental Conditions

Chamber environmental conditions were similar between the LC_{50} and multi-time point blood absorption exposures. Airflow to the exposure chamber during all of the exposures was maintained at 15 L/min/nebulizer for a total of 30 L/min. Chamber temperatures for the exposures ranged from 19°C to 22°C and were slightly lower than the targeted range of 22 ± 2°C. Chamber relative humidity for the exposures ranged from 83 to 90 percent and was considerably higher than the targeted range of 30 to 70 percent. The lower temperature and higher humidity ranges were primarily due to the large volumes of water-based test solution being aerosolized into the exposure chamber. Due to the relatively short duration of the rats to these slightly lower temperatures and higher relatively humidity, the environmental conditions within the exposure chamber were

considered to be acceptable and did not affect the validity of the study. Chamber environmental conditions are summarized in Table 4.

Table 4. Summary of Environmental Conditions in Exposure Chamber

Exposure Mode	Temperature(°C)	Relative Humidity(%)	Airflow(L/min)
LC_{50}	21-22 (n=3)	83-90 (n=3)	30 (n=3)
Multi-Time Point Blood Absorption	19 (n=3)	84-87 (n= 3)	30 (n=3)

Legend:
°C = degrees Centigrade
L/min = liters per minute
n = number of samples collected

7.4 Equivalent Oral Doses

Calculated equivalent oral doses for the gavage portion of the multi-time point blood absorption study, based on body weight and average analytical concentration for the inhalation portion of the study, ranged from 24.2 – 24.6 mg/kg for the male rats and from 34.7 – 38.2 mg/kg for the female rats.

7.5 Mortality and LC_{50} Determination

Five of the 6 animals exposed to 0.18 mg/L NTO for the acute LC_{50} test survived the 4-hour exposure and the recovery period. One male rat was found dead as the animals were being unloaded from the chamber at the conclusion of the 4-hour exposure period and likely died during the last half-hour of the exposure. The animals were observed approximately 10 minutes prior to the end of the exposure and all appeared alive at that time. The Attending Veterinarian observed the animal prior to being submitted for gross necropsy and determined that a number of factors likely caused the death of the animal. These factors included its position in the chamber faceplate during the last 2 hours of the exposure, possible complications involving the flexible barrier used to contain the test material, and low body temperature. The death of this animal was not attributed to the toxicity of the test material and therefore, the 4-hour inhalation median lethal concentration (LC_{50}) of NTO is greater than 0.18 mg/L. All 12 animals exposed, either orally or via inhalation, as part of the multi-time point blood absorption study survived throughout the duration of the test.

7.6 Analysis of Blood Samples

A total of 72 blood samples were analyzed during the multi-time point blood absorption test for the presence of NTO. Forty-two whole blood samples were analyzed for the six rats (seven time points) exposed via inhalation and 30 whole blood samples were analyzed for the six rats (five time points) exposed via oral gavage. The study was originally designed such that the rats exposed via oral gavage would have a total of six blood sampling time points, however the baseline (pre-dosing) blood sample was inadvertently omitted. Since all baseline blood samples analyzed for the rats exposed via inhalation did not detect any pre-exposure NTO in the blood, it was assumed for the purposes of statistical analysis, that all animals exposed via gavage did not as well. Prior to study initiation, AIPH LS personnel determined whole blood to be a sensitive and consistent matrix for determining the absorption of NTO into the bloodstream of rats. The concentration of NTO in the whole blood samples is presented in Appendix F.

7.6.1 Inhalation

The mean whole blood concentration of NTO in male rats exposed via inhalation was 10.2 ± 5.05 micrograms per milliliter (µg/mL) following 1 to 1.1 hours of exposure, 17.7 ± 5.03 µg/mL following 2 to 2.2 hours of exposure, 37.0 ± 6.93 µg/mL following 4 hours of exposure, 5.0 ± 1.82 µg/mL at 4 to 4.1 hours post-exposure, 1.3 ± 1.14 µg/mL at 8 hours post-exposure, and 1.4 ± 1.19 µg/mL at 24 to 24.3 hours post-exposure. The mean whole blood concentration of NTO in female rats exposed via inhalation was 16.0 ± 3.61 µg/mL following 1 hour of exposure, 28.3 ± 2.52 µg/mL following 2 to 2.1 hours of exposure, 46.0 ± 2.00 µg/mL following 3.8 to 4 hours of exposure, 4.5 ± 2.06 µg/mL at 4 to 4.3 hours post-exposure, 1.6 ± 0.38 µg/mL at 8 hours post-exposure, and undetectable at 23.9 to 24.2 hours post-exposure. NTO was not detected in the pre-exposure (baseline) male and female blood samples taken 0.6 to 1.3 hours prior to exposure.

7.6.2 Oral

The mean whole blood concentration of NTO in male rats exposed via oral gavage was 3.9 ± 0.80 µg/mL at 1 to 1.1 hours post-exposure, 2.4 ± 0.31 µg/mL at 2 to 2.1 hours post-exposure, and not detectable 5 to 5.1, 8.3 to 8.4, and 24.1 to 24.2 hours post-exposure. The mean whole blood concentration of NTO in female rats exposed via oral gavage was 8.8 ± 1.26 µg/mL at 1.1 to 1.2 hours post-exposure, 4.6 ± 1.15 µg/mL at 2.1 to 2.2 hours post-exposure, and not detectable 5.2, 8.4 to 8.5, and 24.3 to 24.4 hours post-exposure. Pre-exposure (baseline) male and female blood samples were not obtained prior to oral dose administration.

7.7 Analysis of Urine Samples

The mean urine concentration of NTO in rats exposed via inhalation was 165.3 ± 112.10 µg/mL for male rats and 83.7 ± 50.62 µg/mL for female rats. The mean urine concentration of NTO in rats exposed via oral gavage was 1.1 ± 0.98 µg/mL for male rats and 1.6 ± 1.46 µg/mL for female rats. The concentration of NTO in the urine samples is presented in Appendix G.

7.8 Body Weights of Rats

All surviving rats exposed as part of the LC_{50} inhalation test were weighed on test days 1, 2, 3, 8, 10, and 15. Both surviving male rats exhibited a 5-6 percent body weight loss on post-exposure day 1 while 1/3 of the female rats exhibited a 5 percent body weight loss on post-exposure day 1. All surviving rats exhibited normal weight gain patterns following post-exposure day 1 and experienced an overall weight gain by post-exposure day 8. All rats exposed as part of the multi-time point blood absorption test, both inhalation and gavage, were weighed prior to exposure and on the day following exposure. Male rats exposed via inhalation exhibited a 1-7% body weight loss on post-exposure day 1 while female rats exposed via inhalation exhibited a 1-4% body weight gain. Both male and female rats exposed via oral gavage exhibited a 0-4% body weight gain on post-exposure day 1. The individual body weights for all rats are reported in Appendix H.

7.9 Clinical Observations of Rats

Observation of the rats during both inhalation exposures was somewhat limited due to the thick aerosol test atmosphere. However, to the extent that the rats could be observed during the exposures, compound-stained, wet fur (yellow) was noted on the head, face, and body of the rats. Surviving rats exposed for the LC_{50} test had yellow-stained, wet fur immediately following exposure with the yellow staining persisting throughout the recovery period. One male and one female rat

were lethargic with labored breathing upon removal from the exposure chamber and one rat per sex also exhibited hunched posture. Both surviving male rats had slightly swollen or swollen areas under their necks and one rat per sex exhibited a red-colored nasal discharge. On post-exposure day 1, one female rat had ruffled fur. With the exception of yellow-stained fur, all other clinical signs subsided by post-exposure day 2. All rats exposed for the inhalation portion of the multi-time point blood absorption test had yellow-stained fur on the head/body during the exposure and on post-exposure day 1. Upon removal from the exposure chamber, all animals were cold to the touch with hunched posture and 3/6 rats exhibited a red-colored nasal discharge. No clinical signs were observed for the rats dosed via oral gavage for the multi-time point blood absorption study. The individual clinical signs for all rats are reported in Appendix I.

7.10 Pathology

The one male rat found dead at the conclusion of the LC_{50} test was found to have mild subcutaneous edema in the ventral neck region and a small segment of the jejunum appeared red. The edema was not attributed to exposure to NTO and was believed to be a result of the flexible barrier around the neck of the rat possibly being too tight. In addition, one female rat had a mildly dilated uterus. No other abnormal findings were observed in rats exposed for the LC_{50} test during the gross necropsy. Rats exposed for the multi-time point blood absorption test did not have a gross necropsy performed following the final blood sample. The individual gross necropsy findings for all rats are reported in Appendix J.

7.11 Statistics

The ANOVA performed on the cumulative AUC's indicated that both gender and route of administration were significantly different. Female rats orally dosed with or exposed to equivalent concentrations of NTO had significantly higher NTO blood concentrations compared to male rats (P=.046). Rats exposed to NTO aerosol for a period of 4 hours exhibited significantly higher NTO blood concentrations than those exposed to equivalent doses via oral gavage (P<.001).

8 Discussion

This study was conducted to determine the 4-hour, inhalation median LC_{50} of NTO in male and female rats. Although no inhalation toxicity data existed for NTO prior to this study, previous work had indicated it to be of low acute oral toxicity. For this reason, all method development work was completed with the intention of generating 2 mg/L for an acute inhalation limit test. Under the limit test provision of the EPA test guidelines for materials that are not expected to be acutely toxic via inhalation, a single group of five male and five female rats are exposed to a 2 mg/L test atmosphere for 4 hours. If no lethality is demonstrated at 2 mg/L, no further testing for acute inhalation toxicity is needed (EPA, 1998). Repeated attempts during method development work to generate an NTO aerosol atmosphere of 2 mg/L at the appropriate particle size were unsuccessful and the energetic nature of the test material precluded its generation as a dry particulate. In addition, difficulties with both the generation and chamber distribution of the aerosol atmosphere required that a different faceplate be used. The maximum number of rats that could be exposed at any particular time using this faceplate was six. Therefore, the 4-hour LC_{50} exposure was conducted using three rats per sex at the highest-achievable concentration falling within an acceptable particle size range.

A secondary objective of this study was to determine the effect that two different routes of administration (inhalation and oral) had on the absorption of the chemical into the bloodstream. Previous oral toxicity work performed by this Institute (acute, subacute, and subchronic) confirmed

that NTO was not acutely toxic but did induce male reproductive toxicity upon repeated exposure to higher concentrations (USAPHC (Prov), 2010a). An inhalation OEL was derived from the oral toxicity data, however it was previously unknown if differences existed between the absorption of NTO into the blood through the respiratory and gastrointestinal tracts (USAPHC (Prov), 2010b). It is understood that the previously reported calculation to derive an equivalent oral dose from an inhalation exposure has a number of uncertainties associated with it. First, the amount of NTO retained in the entire respiratory system (α), was assumed to be approximately 90 percent of the analytical chamber concentration. A number of factors, including the chemical properties of the test substance, uptake from the upper respiratory pathways versus the lungs, deposition in the lungs, and the particle size of the generated test material will greatly influence the amount of test material retained in the respiratory system. Every attempt was made to generate the NTO with an MMAD in the range considered respirable for rats (1-4 µm); however, the MMAD for the absorption study was 4.8 µm. The larger particles contained in a test atmosphere are typically retained in the mucous-lined head region and tracheobronchial airways where they may be absorbed or transported out of the respiratory airways via ciliary action. The particles transported out of the airways may then either be expelled or swallowed to the gastrointestinal tract, essentially leading to an additional oral dose. The smaller, soluble particles reaching the alveolar (deep lung) regions may then be absorbed into the blood. Based on the particle size alone, the assumption that 90 percent of the NTO aerosol was retained in the respiratory system may have been a high estimate. However, a certain percentage of larger particles could have been absorbed in the upper respiratory pathways or transported out of the respiratory pathways and swallowed, leading to an increased amount of absorption. Second, an average minute volume of 0.00016 m^3 for rats was used for all animals. Although the resulting variations in the calculated oral dose would be miniscule, the changes in minute volume based on animal size, sex, and respiratory rate were not accounted for in the calculations. Third, the calculation used to determine the equivalent oral dose was not designed to be used with the gavage method of oral administration due to the bolus effect. The rats exposed via inhalation for the multi-time point blood absorption test were exposed to a consistent concentration for a period of 4 hours while rats exposed via oral gavage received the entire dose at one time. Obviously differences in the method of dose administration will lead to differences in the blood concentration/time curves; however, gavage is still one of the most accurate methods to administer an oral dose. Alternative methods to slowly introduce the test substance into the rats (i.e., timed injection) would have involved the introduction of a different route of exposure and the blood concentrations resulting from an oral dose were most desirable since the current OEL was extrapolated from the results of an oral subchronic study. Mixing the test substance in animal feed was not considered an option for this study due to the inaccuracy of the dose (i.e., food spillage) and difficulties ensuring that the animals consumed all of the food within a specified period of time.

Given the inherent limitations of comparing the whole blood concentrations of a test material introduced by two different exposure routes, acute exposure to NTO via inhalation does appear to induce higher blood concentrations in laboratory rats compared to those exposed via oral gavage. Cumulative area-under-curve blood values averaged 206.5 ± 31.62 µg/mL for rats exposed by inhalation and 13.4 ± 5.25 µg/mL for rats exposed by oral gavage. NTO blood concentrations peaked at the sampling time point immediately following exposure for both the inhalation and oral routes. These concentrations then dropped below the limit of detection by 5 hours post-exposure for orally-dosed animals while blood concentrations of 5/6 animals exposed via inhalation remained above the limit of detection at 8 hours post-exposure. Two of the six animals exposed via inhalation still had blood concentrations above the limit of detection 24 hours following exposure. The longer elimination times observed for these animals could be due to a number of factors including higher peak NTO blood concentrations, additional oral intake during preening, or additional absorption from the large NTO aerosol particles retained in the nasal passages following the exposure period.

Although the NTO whole blood concentrations observed between male and female rats exposed via inhalation and oral gavage were statistically compared, the fact that male and female rats are typically exposed simultaneously to the same test concentration during acute inhalation studies likely led to the elevated female blood concentrations compared to the males. By study design, the female rats essentially receive a higher dose per body weight since they are exposed to the same test atmosphere concentration. The calculation used for converting an inhalation dose to an oral dose does consider body weight but with the males and females exposed to identical inhalation atmospheres, the conversion calculation also yields a higher oral mg/kg dose.

9 Conclusions

Rats were exposed nose-only to a 0.18 mg/L aerosol atmosphere of NTO for a single 4-hour exposure. No test compound-related mortalities occurred in rats exposed during the study and no adverse toxic signs, body weight changes, or gross necropsy findings were observed in exposed rats. The results of the LC_{50} portion of this study indicate that acute inhalation exposure to the highest-achievable concentration of NTO aerosol (0.18 mg/L) is relatively nontoxic to rats.

The results of the multi-time point blood absorption portion indicated that, under the stated study conditions and limitations, acute exposure to NTO via inhalation appears to induce higher whole blood concentrations in laboratory rats compared to those exposed via oral gavage.

10 Point of Contact

Questions pertaining to this report should be referred to Lee C.B. Crouse at DSN 584-5088, Commercial 410-436-5088, or by e-mail: lee.crouse.civ@mail.mil

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Appendix A

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Appendix B

QUALITY ASSURANCE STATEMENT

For: Toxicology Study No. 85-XC-0ENTb-11, Protocol No. 0ENT-24-11-07-04, Acute Inhalation Toxicity and Blood Absorption of 3-Nitro-1,2,4-Triazol-5-One (NTO) in Rats, the following critical phases were inspected/audited by the Quality Systems Office (QSO):

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Study Protocol Good Laboratory Practice Standards and Animal Care Review	05/26/2011	05/26/2011

Critical Phase Inspected/Audited	Date Inspected /Audited	Date Reported to Management/SD
Compliance with Protocol Modification-Pilot Study	11/07/2011	11/16/2011
Administration of Test Substance by Inhalation	11/21/2011	11/29/2011
LC50/Rangefinding-Analysis of the Test Atmosphere	11/21/2011	11/29/2011
Test System - Facilities, Identification, Husbandry & Food and Water Supply	11/22/2011	11/30/2011
Administration of Test Substance by Oral Gavage	11/22/2011	11/30/2011
Post Oral Gavage Blood Collection Procedures	11/22/2011	11/30/2011
Multi-Timepoint Blood Absorption Test-Inhalation Procedures	12/01/2011	12/13/2011
Experiment # 3 - Raw Data Documentation Procedures	12/01/2011	12/13/2011
Exp 3 - Catheter Post Gavage Blood Collection	12/02/2011	12/14/2011
Exp 3-Urine Collection/Compliance w/ Study Modification # 2	12/02/2011	12/14/2011
In-Life Study Endpoint Criteria	12/05/2011	12/22/2011
Final Study Report Review	05/17/2013	05/21/2013
Study Raw Data Review	05/17/2013	05/21/2013

Note: All findings were made known to the Study Director and the Program Manager at the time of the audit/inspection. If there were no findings during the inspection, the inspection was reported to Management and the Study Director on the date shown in the table. A review of the raw data and records indicates the above mentioned report accurately reflects the raw data and study as it was conducted.

Michael P. Kefauver
GLP Quality Assurance Specialist, QSO

09/13/2013
Date

Appendix C

Archives and Study Personnel

1. ARCHIVES.

a. All raw data, documentation, records, protocol, and a copy of the final report generated as a result of this study will be archived in room 1026, Building E-2100, USAPHC, for a minimum of five (5) years following submission of the final report to the Sponsor.

b. Records on animal receipt, diet, and facility environmental parameters will be archived by the Veterinary Medical Division, Toxicology Portfolio, for a minimum of five (5) years following submission of the final report to the Sponsor.

c. Some ancillary records pertaining to this study, such as instrument maintenance logs, animal room observation logs, etc., will not be archived until those logbooks have been completed. Once complete they will be archived in room 1026, Building E-2100, USAPHC.

d. Wet tissues, histology slides, and paraffin blocks are stored in building E-5158.

2. PERSONNEL.

a. Management

(1) Management (In-Life): COL Chris E. Hanson, Portfolio Director, Toxicology; Glenn J. Leach, Ph.D., Program Manager, Toxicity Evaluation Program (TEP); Dr. Mark S. Johnson, Ph.D., Program Manager, Health Effects Research Program (HERP).

(2) Management (Report): Mark S. Johnson, Portfolio Director, Toxicology; Arthur J. O'Neill, Program Manager (Acting), TEP; Dr. Mark S. Johnson, Ph.D., Program Manager, HERP.

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i. Archivist: Martha Thompson, Data Acquisition Specialist, TEP.

Appendix D

Chamber Distribution

APPENDIX D
Protocol No. 0ENT-24-11-07-04
Acute Inhalation Toxicity and Blood Absorption of NTO in Rats

Chamber Distribution of NTO Atmosphere

Location of Sample Port	Sample #	Concentration of NTO (mg/L)
Reference Faceplate Port	1	0.173
Reference Faceplate Port	3	0.170
Reference Faceplate Port	5	0.189
Reference Faceplate Port	7	0.195
Reference Faceplate Port	9	0.170
	Mean	**0.179**
Top of Faceplate	2	0.137
Middle Center of Faceplate	4	0.236
Middle Right of Faceplate	6	0.189
Bottom of Faceplate	8	0.126
	Mean	**0.172**
Total of 9 Samples	**Mean**	**0.176**

The reference faceplate port was designated as the middle left port of the faceplate

Confirmation of Uniform Distribution of NTO Aerosol Within Exposure Chamber

Top of Faceplate	0.137 mg/L / 0.172 mg/L = 80%
Middle Center of Faceplate	0.236 mg/L / 0.172 mg/L = 137%
Middle Right of Faceplate	0.189 mg/L / 0.172 mg/L = 110%
Bottom of Faceplate	0.126 mg/L / 0.172 mg/L = 73%

The top and bottom ports of the faceplate were not used for the animal exposures due to the lack of aerosol uniformity.

Confirmation of Correlation Between Reference Sample Port and Animal Exposure Ports

Reference Faceplate Port Sample #1	0.173 mg/L / 0.172 mg/L = 101%
Reference Faceplate Port Sample #2	0.170 mg/L / 0.172 mg/L = 99%
Reference Faceplate Port Sample #3	0.189 mg/L / 0.172 mg/L = 110%
Reference Faceplate Port Sample #4	0.195 mg/L / 0.172 mg/L = 113%
Reference Faceplate Port Sample #5	0.170 mg/L / 0.172 mg/L = 99%

Appendix E

Exposure Chamber Atmospheric Concentration

APPENDIX E
Protocol No. 0ENT-24-11-07-04
Acute Inhalation Toxicity and Blood Absorption of NTO in Rats

Exposure Chamber Atmospheric Concentration

Acute LC_{50} Test

Sample #	NTO Aerosol Concentration in Exposure Chamber (mg/L)
1	0.208
2	0.166
3	0.159
4	0.181
5	0.171
6	0.188
7	0.218
8	0.190
9	0.178
Mean ± S.D.	**0.1843 ± 0.01919**

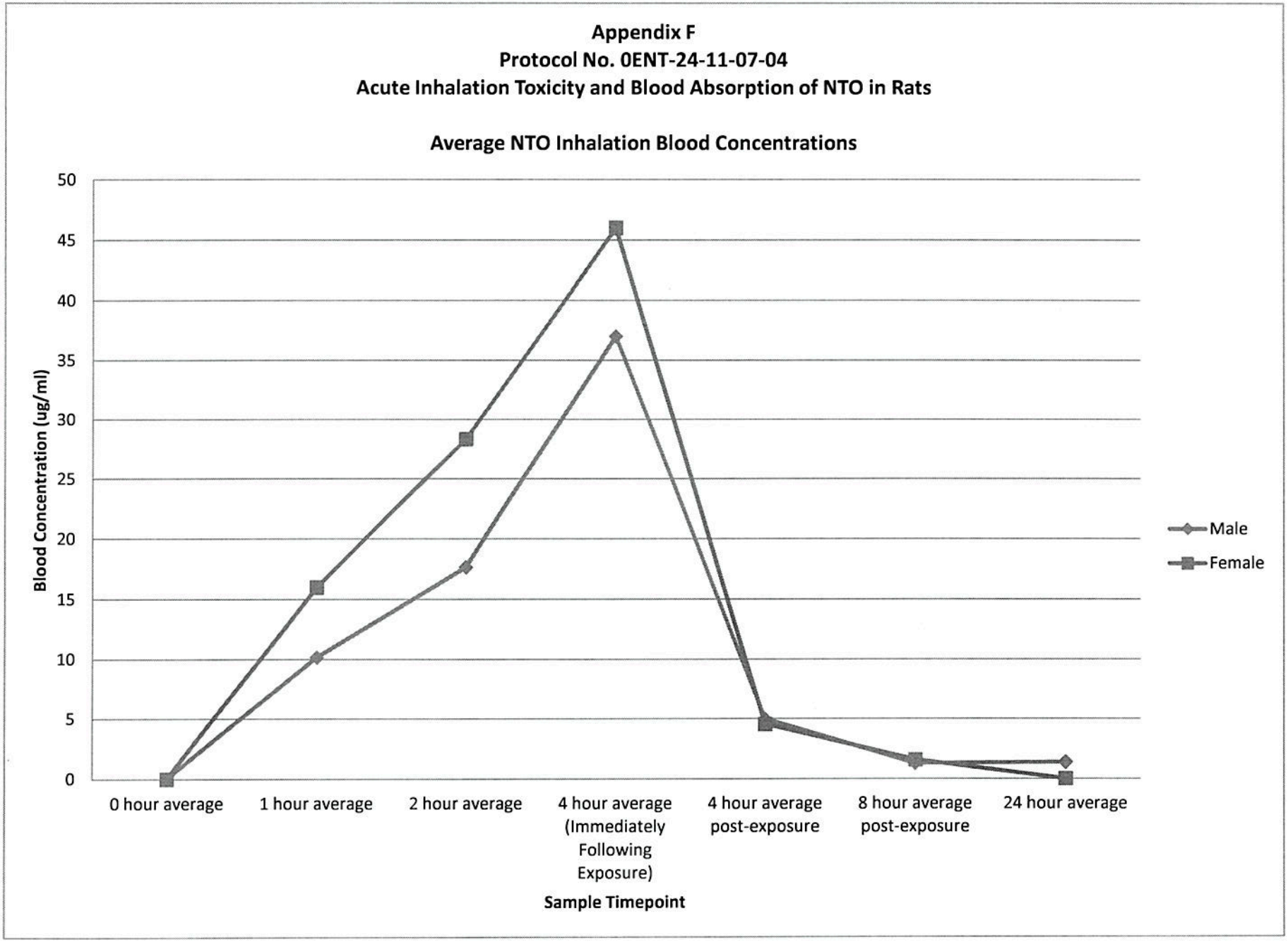
Multi-Time Point Blood Absorption Test

Sample #	NTO Aerosol Concentration in Exposure Chamber (mg/L)
1	0.193
2	0.209
3	0.177
4	0.215
5	0.189
6	0.215
7	0.222
8	0.186
9	0.189
10	0.223
11	0.276
Mean ± S.D.	**0.2085 ± 0.02744**

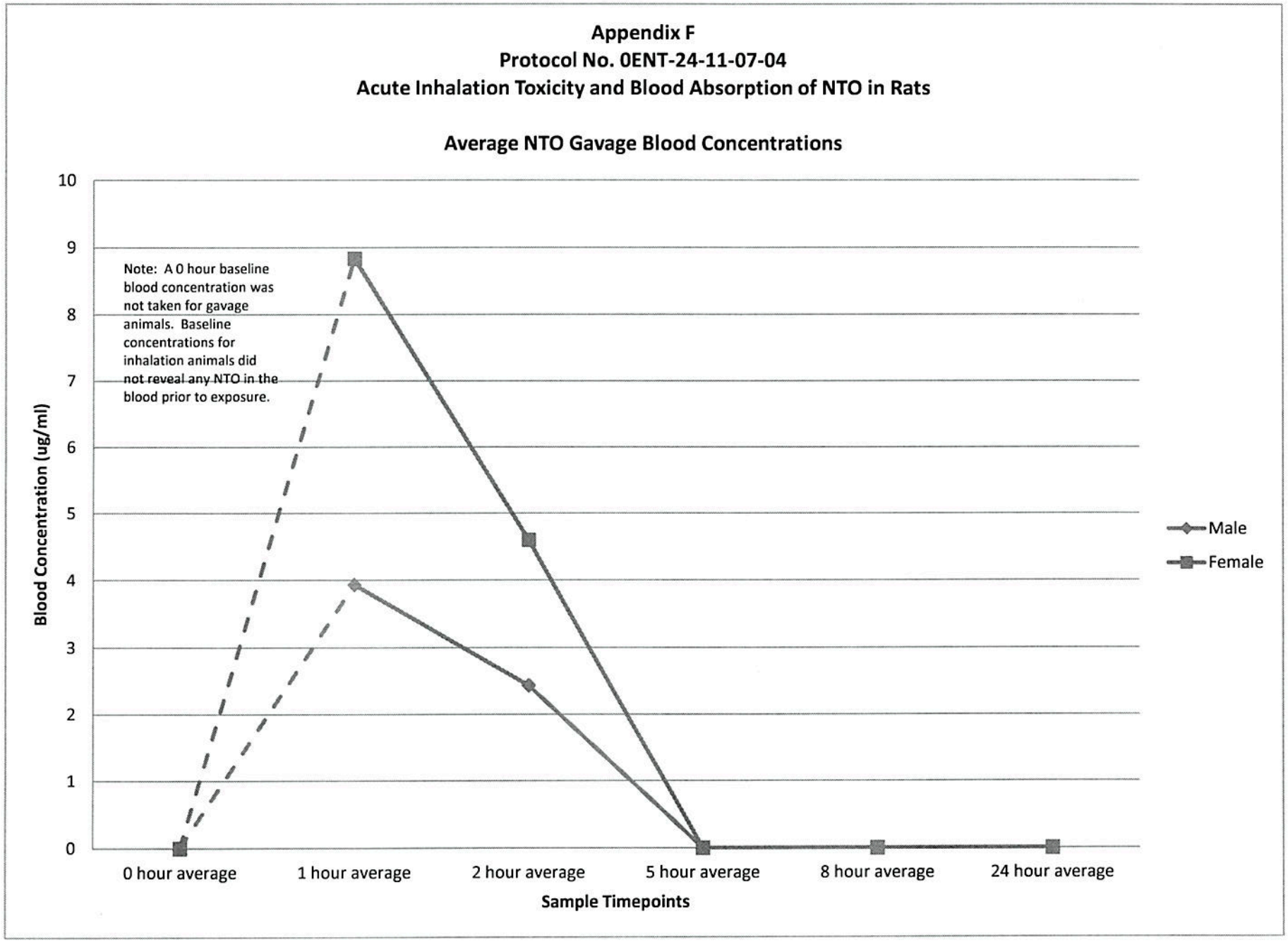
Appendix F

Blood Concentrations

Appendix F
Protocol No. 0ENT-24-11-07-04
Acute Inhalation Toxicity and Blood Absorption of NTO in Rats



Appendix F
Protocol No. 0ENT-24-11-07-04
Acute Inhalation Toxicity and Blood Absorption of NTO in Rats



APPENDIX F
Protocol No. 0ENT-24-11-07-04
Acute Inhalation Toxicity and Blood Absorption of NTO in Rats

Individual Blood Concentrations For Multi-Time Point Blood Absorption Test

Inhalation

Animal #	Sex	Exposure Level	NTO Blood Concentrations (ug/ml) 0 Hour (Baseline)	1 Hour	2 Hour	4 Hour[a]	8 Hour[b]	12 Hour[c]	24 Hour[d]
12-0058	Male	.208 mg/L	0	7.3	17	45	6.9	2.2	2.1
12-0059	Male	.208 mg/L	0	7.2	13	33	4.7	1.6	2
12-0060	Male	.208 mg/L	0	16	23	33	3.3	0	0
12-0062	Female	.208 mg/L	0	12	26	46	6.9	1.4	0
12-0063	Female	.208 mg/L	0	19	28	44	3.2	2	0
12-0064	Female	.208 mg/L	0	17	31	48	3.5	1.3	0
		Mean	**0**	**13.08**	**23.00**	**41.50**	**4.75**	**1.42**	**0.68**
		S.D.	**0**	**5.061**	**6.841**	**6.716**	**1.750**	**0.776**	**1.059**

a. Represents blood samples taken immediately following removal from exposure chamber.
b. Represents blood samples taken 4 hours following inhalation exposure.
c. Represents blood samples taken 8 hours following inhalation exposure.
d. Represents blood samples taken the following morning after inhalation exposure.

Oral Gavage

Animal #	Sex	Equivalent Oral Dose	NTO Blood Concentrations (ug/ml) 0 Hour (Baseline)	1 Hour	2 Hour	5 Hour	8 Hour	24 Hour
12-0055	Male	24.2 mg/kg	ND	4.7	2.7	0	0	0
12-0056	Male	24.6 mg/kg	ND	3.1	2.1	0	0	0
12-0057	Male	24.5 mg/kg	ND	4	2.5	0	0	0
12-0065	Female	34.7 mg/kg	ND	9	5.5	0	0	0
12-0066	Female	38.2 mg/kg	ND	7.5	5	0	0	0
12-0067	Female	35.6 mg/kg	ND	10	3.3	0	0	0
		Mean		**6.38**	**3.52**	**0**	**0**	**0**
		S.D.		**2.845**	**1.406**	**0**	**0**	**0**

ND. No data. For statistical purposes baseline samples were assumed to be 0 based on baseline results from inhalation portion.

APPENDIX F (cont)
Protocol No. 0ENT-24-11-07-04
Acute Inhalation Toxicity and Blood Absorption of NTO in Rats

Summary of Blood Concentrations For Multi-Time Point Blood Absorption Test

Inhalation

Sex	Exposure Level		NTO Blood Concentrations (ug/ml) 0 Hour (Baseline)	1 Hour	2 Hour	4 Hour[a]	8 Hour[b]	12 Hour[c]	24 Hour[d]
Male	.208 mg/L	Mean	0	10.17	17.67	37.00	4.97	1.27	1.37
		S.D.	0	5.052	5.033	6.928	1.815	1.137	1.185
Female	.208 mg/L	Mean	0	16.00	28.33	46.00	4.53	1.57	0.00
		S.D.	0	3.606	2.517	2.000	2.055	0.379	0.000

a. Represents blood samples taken immediately following removal from exposure chamber.
b. Represents blood samples taken 4 hours following inhalation exposure.
c. Represents blood samples taken 8 hours following inhalation exposure.
d. Represents blood samples taken the following morning after inhalation exposure.

Oral Gavage

Sex	Average Equivalent Oral Dose		NTO Blood Concentrations (ug/ml) 0 Hour (Baseline)	1 Hour	2 Hour	5 Hour	8 Hour	24 Hour
Male	24.4 mg/kg	Mean	ND	3.93	2.43	0.00	0.00	0.00
		S.D.	ND	0.802	0.306	0.000	0.000	0.000
Female	36.2 mg/kg	Mean	ND	8.83	4.60	0.00	0.00	0.00
		S.D.	ND	1.258	1.153	0.000	0.000	0.000

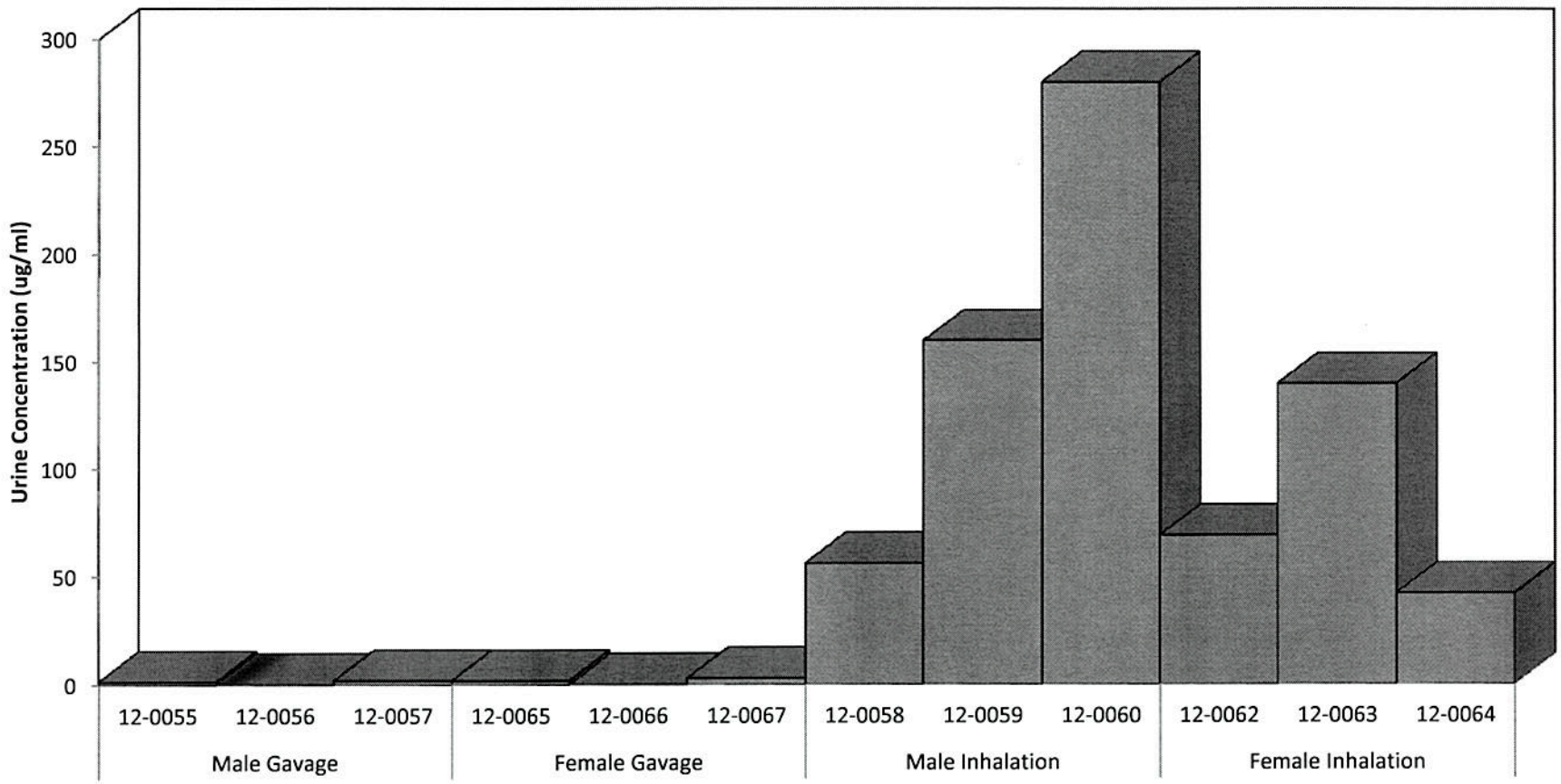
ND. No data. For statistical purposes baseline samples were assumed to be 0 based on baseline results from inhalation portion.

Appendix G

Urine Concentrations

Appendix G
Protocol No. 0ENT-24-11-07-04
Acute Inhalation Toxicity and Blood Absorption of NTO in Rats

NTO Urine Concentrations Gavage vs. Inhalation



Note: Rats were placed in metabolism cages overnight on the day of exposure and collected the following morning for analysis for both gavage and inhalation

APPENDIX G
Protocol No. 0ENT-24-11-07-04
Acute Inhalation Toxicity and Blood Absorption of NTO in Rats

Individual Urine Concentrations For Multi-Time Point Blood Absorption Test

Inhalation

Animal #	Sex	Exposure Level	NTO Urine Concentration (ug/ml)
12-0058	Male	.208 mg/L	56
12-0059	Male	.208 mg/L	160
12-0060	Male	.208 mg/L	280
12-0062	Female	.208 mg/L	69
12-0063	Female	.208 mg/L	140
12-0064	Female	.208 mg/L	42
		Mean	**124.50**
		S.D.	**89.732**

Oral Gavage

Animal #	Sex	Equivalent Oral Dose	NTO Urine Concentration (ug/ml)
12-0055	Male	24.2 mg/kg	1.4
12-0056	Male	24.6 mg/kg	0
12-0057	Male	24.5 mg/kg	1.9
12-0065	Female	34.7 mg/kg	1.8
12-0066	Female	38.2 mg/kg	0
12-0067	Female	35.6 mg/kg	2.9
		Mean	**1.33**
		S.D.	**1.145**

Appendix H

Body Weights

APPENDIX H
Protocol No. 0ENT-24-11-07-04
Acute Inhalation Toxicity and Blood Absorption of NTO in Rats

Individual Body Weights (grams)

Acute LC_{50} Test

Sex	Animal ID	Day 1	Day 2	Day 3	Day 8	Day 10	Day 15
	12-0032	246	(f)	(f)	(f)	(f)	(f)
Male	12-0033	238	226	237	277	291	321
	12-0034	241	226	240	288	309	343
	Mean	**241.7**	**226.0**	**238.5**	**282.5**	**300.0**	**332.0**
	SD	**4.04**	**0.00**	**2.12**	**7.78**	**12.73**	**15.56**
	12-0036	195	200	201	202	223	228
Female	12-0038	183	185	185	205	207	222
	12-0039	194	185	195	217	222	246
	Mean	**190.7**	**190.0**	**193.7**	**208.0**	**217.3**	**232.0**
	SD	**6.66**	**8.66**	**8.08**	**7.94**	**8.96**	**12.49**

(f) = Animal died on study.

Multi-Timepoint Blood Absorption Test

Sex	Exposure Method	Animal ID	Day 1	Day 2
	Gavage	12-0055	297	299
Male	Gavage	12-0056	292	299
	Gavage	12-0057	293	302
		Mean	**294.0**	**300.0**
		SD	**2.65**	**1.73**
	Inhalation	12-0058	254	237
Male	Inhalation	12-0059	279	277
	Inhalation	12-0060	272	266
		Mean	**268.3**	**260.0**
		SD	**12.90**	**20.66**
	Gavage	12-0065	207	215
Female	Gavage	12-0066	188	188
	Gavage	12-0067	202	207
		Mean	**199.0**	**203.3**
		SD	**9.85**	**13.87**
	Inhalation	12-0062	219	221
Female	Inhalation	12-0063	203	206
	Inhalation	12-0064	197	205
		Mean	**206.3**	**210.7**
		SD	**11.37**	**8.96**

Appendix I

Clinical Observations

APPENDIX I
Protocol No. 0ENT-24-11-07-04
Acute Inhalation Toxicity and Blood Absorption of NTO in Rats

Individual Clinical Signs

Acute LC_{50} Test

Sex	Animal ID	Observation	First Day Observed	Last Day Observed
Male	12-0032	Found Dead	1	1
	12-0033	Hunched Posture	1	1
		Yellow-Stained and Wet Fur on Head/Body	1	1
		Yellow-Stained Fur on Head/Body	1	15
		Slight Swollen Area Under Neck	1	1
		Terminal Sacrifice 12/5/11		
	12-0034	Lethargic	1	1
		Yellow-Stained and Wet Fur on Head/Body	1	1
		Labored Breathing	1	1
		Swollen Area Under Neck	1	1
		Yellow-Stained Fur on Head/Body	1	15
		Lung Noise	1	1
		Red-Colored Discharge Around Nose	1	1
		Terminal Sacrifice 12/5/11		
Female	12-0036	Lethargic	1	1
		Yellow-Stained and Wet Fur on Head/Body	1	1
		Labored Breathing	1	1
		Hunched Posture	1	1
		Yellow-Stained Fur on Head/Body	1	15
		Ruffled Fur	2	2
		Terminal Sacrifice 12/5/11		
	12-0038	Yellow-Stained and Wet Fur on Head/Body	1	1
		Yellow-Stained Fur on Head/Body	1	15
		Terminal Sacrifice 12/5/11		
	12-0039	Hunched Posture	1	1
		Yellow-Stained and Wet Fur on Head/Body	1	1
		Yellow-Stained Fur on Head/Body	1	15
		Red-Colored Discharge Around Nose	1	1
		Terminal Sacrifice 12/5/11		

Multi-Time Point Blood Absorption Test

Sex/ Exposure Method	Animal ID	Observation	First Day Observed	Last Day Observed
Male/Gavage	12-0055	Terminal Sacrifice 12/3/11		
	12-0056	Terminal Sacrifice 12/3/11		
	12-0057	Terminal Sacrifice 12/3/11		
Male/Inhalation	12-0058	Yellow-Stained Fur on Head/Body	2	2
		Terminal Sacrifice 12/2/11		
	12-0059	Yellow-Stained Fur on Head/Body	2	2
		Terminal Sacrifice 12/2/11		
	12-0060	Yellow-Stained Fur on Head/Body	2	2
		Terminal Sacrifice 12/2/11		
Female/Gavage	12-0065	Terminal Sacrifice 12/3/11		
	12-0066	Terminal Sacrifice 12/3/11		
	12-0067	Terminal Sacrifice 12/3/11		
Female/Inhalation	12-0062	Yellow-Stained Fur on Head/Body	2	2
		Terminal Sacrifice 12/2/11		
	12-0063	Yellow-Stained Fur on Head/Body	2	2
		Terminal Sacrifice 12/2/11		
	12-0064	Yellow-Stained Fur on Head/Body	2	2
		Terminal Sacrifice 12/2/11		

Appendix J

Necropsy Findings

APPENDIX J
Protocol No. 0ENT-24-11-07-04
Acute Inhalation Toxicity and Blood Absorption of NTO in Rats

Gross Pathological Observations

Acute LC_{50} Test

Sex	Animal ID	Gross Observations
Male	12-0032	Wet hair coat
		Mild subcutaneous edema ventral neck predominantly surrounding the salivary glands
		Small segment of jejenum appears red on cut surface; mucosa appears normal
	12-0033	Moderate yellow staining dorsal head and body
	12-0034	Moderate yellow staining dorsal head and body
Female	12-0036	Moderate yellow staining dorsal head and body
		Mildly dilated uterus
		Mild amount of bedding in stomach
	12-0038	Moderate yellow staining dorsal head and body
	12-0039	Moderate yellow staining dorsal head and body

Appendix K

Study Protocol with Modifications

ANIMAL USE PROTOCOL
TOXICOLOGY PORTFOLIO
ARMY INSTITUTE OF PUBLIC HEALTH
U.S. ARMY PUBLIC HEALTH COMMAND
ABERDEEN PROVING GROUND, MD 21010-5403

PROTOCOL TITLE: Acute Inhalation Toxicity and Blood Absorption of 3-Nitro-1,2,4-Triazol-5-One (NTO) in Rats

PROTOCOL NUMBER: ØENT-24-11-Ø7-Ø4

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Environmental Acquisition and Logistics Sustainment Program (EASLP)
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I. NON-TECHNICAL SYNOPSIS

The acute toxicity of 3-nitro-1,2,4-triazol-5-one (NTO), an insensitive, energetic material used in explosive formulations, will be evaluated by conducting a series of three experiments/tests. The first test will determine the acute inhalation toxicity (LC_{50}) of the test substance. Groups of 10 rats each will be exposed to a single, 4-hour atmospheric concentration of the test substance in air. An evaluation of the acute inhalation toxicity data will determine the relationship, if any, between the exposure of the rats to the test substance and the incidence and severity of abnormalities, including behavioral and clinical abnormalities, the reversibility of observed abnormalities, gross lesions, body weight changes, effects on mortality, and any other toxic effects. The second test, conducted in conjunction with the acute inhalation test, will serve as a rangefinding tool, and will collect a single, terminal blood sample from a small number of rats following dosage of the test substance by either the inhalation or oral gavage route of administration. The data generated from the rangefinding study will be used to select appropriate dosage concentrations for the multi-timepoint blood sample test. The third test will collect blood samples at multiple timepoints from 12 catheterized rats to determine the effect that two different routes of administration (inhalation and oral) have

on the absorption of the chemical into the blood. All rats will be monitored throughout the study for body weights and clinical signs. The acute inhalation test rats will be euthanized and receive a gross necropsy following the 14-day recovery period. Rats from the rangefinding test will be anesthetized and have blood samples collected prior to euthanasia. Rats from the multi-timepoint blood absorption test will be euthanized following collection of the final blood sample. Regulatory test guidelines typically state that the rat is the preferred species for this type of study. Historically, rats have been used for acute inhalation and oral toxicity studies and therefore are the recommended species due to the extensive historical database.

II. BACKGROUND

II.1. Background: 3-Nitro-1,2,4-triazol-5-one (NTO) is being investigated as a less sensitive direct replacement for traditional explosives such as TNT and RDX. NTO is a crystalline powder that is one of the components used in the formulation of an insensitive explosive referred to as IMX101. NTO was first reported in 1905, but was not used as an explosive until the early 1980's when it was discovered that the French were developing a "new insensitive explosive", which was later reported to be NTO. Renewed interest in the energetic properties of NTO has been fueled by the need to develop munitions that are less prone to inadvertent initiation during transport and routine handling. The reduced sensitivity to environmental stimuli and nearly equal performance during testing make NTO-based formulations desirable replacements for currently fielded munitions (references 1 and 2). As a potential new component of munitions formulations, NTO must not only meet certain performance criteria, but must also be acceptable from the perspective of human health and the environment. To ensure its safe use by military personnel and production employees handling the material on a daily basis, the toxicity of NTO must be investigated. Toxicological testing will be conducted by the U.S. Army Public Health Command (Provisional) (USAPHC (Prov)), Portfolio of Toxicology (TOX).

II.2. Literature Search for Duplication

II.2.1. Literature Source(s) Searched: AGRICOLA, BRD, DTIC, FEDRIP, NTIS, TOXLINE, PubMed, Web of Science

II.2.2. Date of Search: 08 March 2011

II.2.3. Period of Search: 1900-2011

II.2.4. Key Words of Search: 3-nitro-1,2,4-triazol-5-one, nitro compounds, triazoles, nto, aerosol, inhalation, breath, lung, pulmonary, respiration, toxicity, blood, concentration, rats, mice, mouse, rodent, guinea pigs, animals

II.2.5. Results of Search: A total of 86 references resulted from the literature search that was performed for NTO. However, no inhalation toxicity studies or blood absorption studies for NTO were found that would suggest that this study would be a duplicate effort. As such, the present study is not a duplication of the information available in the literature.

III. OBJECTIVE/HYPOTHESIS

The primary objective of this study is to determine the acute inhalation toxicity of NTO. Rats will be exposed to atmospheres of NTO and an estimate of the inhalation median lethal concentration (LC_{50}) will be determined. The LC_{50} is defined as the calculated atmospheric concentration of test substance expected to cause death in 50% of exposed animals either on the day of exposure or within at least 14 days post exposure. The secondary objective of this study will be to determine the effect that two different routes of administration (inhalation and oral) have on the absorption of the chemical into the blood.

IV. MILITARY RELEVANCE

As a result an initiative by the Department of Defense (DOD) to improve munitions safety, the US Army is developing insensitive munitions (IM) for incorporation into its inventory of conventional military munitions systems. The Army's IM Program is dedicated to developing munitions that reliably perform as they are intended but are less prone to inadvertent initiation from external stimuli such as bullet/fragment impact, heat from fire, and shock from neighboring explosions (reference 3). The production of insensitive munitions requires the use of intrinsically less sensitive explosives that contribute to lower order responses to inadvertent external stimuli. Despite the slightly lower performance of NTO compared to TNT, there has been a renewed interest in NTO use in explosive formulations based on its lower sensitivity as a melt-cast medium observed during testing and the less stringent shipping requirements. This has led to the development of a range of melt-castable explosives at Picatinny Arsenal, collectively known as "PAX" explosives (reference 4). To support possible fielding of these PAX explosives, a Toxicity Clearance would have to be granted and occupational exposure guidelines developed. Consequently, toxicity data in a mammalian system need to be generated to assess occupational health hazards associated with the use and production of this material.

V. MATERIALS AND METHODS

V.1. Experimental Design and General Procedures: This study consists of three experiments in which the toxicity of NTO will be evaluated in rats: an acute inhalation toxicity test (LC_{50}), a rangefinding blood absorption test, and a multi-timepoint blood absorption test. For the acute inhalation toxicity test, group(s) of 5 male and 5 female rats will be exposed (nose-only) for a single, 4-hour period to an atmosphere of the test substance in air. Multiple inhalation exposures may be conducted (each with a different group of rats) in order to determine the LC_{50}. Following the recovery period, surviving rats will receive a gross necropsy. For the inhalation phase of a rangefinding blood absorption test, groups of 2 rats each will be exposed by inhalation to different concentrations of NTO (single exposure; nose-only, one male and one female), and following exposure, rats will be anesthetized (isoflurane or CO_2 gas) and a single terminal blood sample (approximately 3-6 ml) will be collected via cardiac puncture. Immediately following blood collection, rats will be euthanized by CO_2 (euthanasia will be ensured by pneumothorax). An additional component of the rangefinding blood absorption test will consist of groups of 2 rats each being dosed orally (single dose;

gavage; one male and one female). Following the oral dose, all rats will be anesthetized (isoflurane or CO_2 gas) and a single terminal blood sample (approximately 6 ml) will be collected via cardiac puncture. Immediately following blood collection, rats will be euthanized by CO_2 (euthanasia will be ensured by pneumothorax). For the multi-timepoint blood absorption test, one group of 12 rats, each fitted with a subcutaneous femoral artery catheter, will either be exposed to NTO by inhalation (single exposure; nose-only; 3 male and 3 female) or dosed orally (single dose; gavage; 3 male and 3 female). The absorption of NTO will be determined from blood samples collected from each rat via the catheter at up to 7 selected timepoints during the test. Immediately following the final blood collection sample, each rat will be euthanized by injection of a solution of sodium pentobarbital into the catheter and euthanasia will be ensured by pneumothorax. All rats will be monitored throughout the study for mortality/moribundity, body weights, and/or clinical signs. Estimated initiation date for the study is July 2011. Estimated completion date for the study is September 2011.

V.1.1. Experiment 1: Acute Inhalation Toxicity (LC_{50}) Test

In an attempt to determine the acute toxicity associated with inhalation exposure to NTO, groups of 10 rats each (5 male and 5 female) will be exposed nose-only to a single, 4-hour atmosphere of the test substance in air. Following exposure, surviving rats will be retained for at least a 14-day recovery period. In the event that significant signs of toxicity (e.g., mortality, neurotoxicity, etc.) are delayed, the duration of the recovery period may be extended in order to determine the length of time for recovery, however, the recovery period will not exceed 28 days. During the test, rats will be monitored for morbidity/mortality, weight loss, and/or clinical signs of toxicity. Rats that survive the recovery period will be euthanized by CO_2 (euthanasia will be ensured by pneumothorax) and then necropsied. All rats will receive a gross necropsy. Generally for acute inhalation toxicity studies, if no mortality occurs in exposed animals at a limit concentration (2000 mg/m^3) of the test substance being tested, only one exposure is required. However, since it is unknown if this acute inhalation test with NTO will be a limit test, multiple exposures with additional groups of animals may need to be conducted following the initial exposure. These subsequent exposures will be conducted at concentrations appropriately adjusted based on the results of previous exposure(s). Up to a total of 5 exposures may need to be conducted in order to determine the LC_{50} for NTO. If one sex is observed to be more sensitive to the toxicological properties of the test substance, subsequent exposures with 10 rats of the more sensitive sex may be conducted. The primary endpoint of this study is mortality attributed to exposure of the test substance. The number of rats per group that expire either during the exposure or during the recovery period constitutes the fractional mortality for each exposure. In an attempt to minimize potential issues related to the shipment of rats that are not within the desired age or weight ranges, one additional rat per sex will be ordered for each exposure to ensure that each exposure is initiated with 5 male and 5 female rats within the proper age and weight ranges. A total of 12 rats will be ordered for each exposure with a total of 10 rats actually exposed. A maximum of 5 exposures will be conducted. Each of the "additional" animals not used for the inhalation exposure will be used for the rangefinding work to be conducted in Experiment 2. However, if for some reason the decision is made not to use these "additional" animals for the rangefinding work, they will be transferred to another

protocol or humanely euthanized per protocol guidance. Details of the experimental design and general procedures for an acute inhalation toxicity study are described in TOX SOP 029 (reference 5), with modifications and clarifications detailed in this protocol.

Experiment 1. Acute Inhalation Toxicity (LC_{50}) Test

Exposure No. / Design Concentration	No. of Male Rats (a)	No. of Female Rats (a)
1. 2 mg/L or TBD	5 + 1 = 6	5 + 1 = 6
2. TBD	5 + 1 = 6	5 + 1 = 6
3. TBD	5 + 1 = 6	5 + 1 = 6
4. TBD	5 + 1 = 6	5 + 1 = 6
5. TBD	5 + 1 = 6	5 + 1 = 6
	TOTAL = 30 (a)	**TOTAL = 30 (a)**

(a) Five rats/sex will be used for each exposure. One additional rat/sex will be ordered for weight matching purposes. Each of the rats designated as "additional" for Experiment 1 will be used for the rangefinding test in Experiment 2 (see below).

V.1.2. Experiment 2: Rangefinding Blood Absorption Test

A rangefinding blood absorption test will be conducted to determine the effect that different inhalation concentrations of NTO and the route of dose administration has on the absorption of NTO into the blood of rats. The data generated from the rangefinding test will be used to select appropriate dosage concentrations for the multi-timepoint blood absorption test (Experiment 3). The effect that varying concentrations of NTO has on the absorption of NTO into the blood of rats will be examined by exposing groups of 2 rats each (one male and one female) to different concentrations of NTO atmospheres. For each inhalation exposure, the 2 rats designated as "additional" from the acute inhalation exposure (Experiment 1) will be exposed nose-only to a single, 4-hour atmosphere of the test substance concurrently with the 10 rats being exposed in the acute inhalation exposure (Experiment 1). Following the exposure, (e.g., within 30 minutes), these 2 rats will be anesthetized (isoflurane or CO_2 gas) and a single blood sample (approximately 3-6 ml) will be collected via cardiac puncture. The effect that the route of administration has on the absorption of NTO into the blood of rats will be examined by orally dosing (gavage) one group of rats (one male and one female) to a selected concentration of NTO at a dose considered similar to one of the inhalation exposure concentrations conducted during the acute inhalation test (Experiment 1). Following administration of the dose (e.g., within 1 hour), these 2 orally dosed rats will be anesthetized (isoflurane or CO_2 gas) and a single blood sample (approximately 3-6 ml) will be collected via cardiac puncture. Immediately following blood collection, all rats will be euthanized by CO_2 and euthanasia will be ensured by pneumothorax. All blood samples will be collected and evaluated per TOX SOP 053 (reference 6). Blood samples will immediately be injected into a vessel containing a measured volume of solvent specified by Army Institute of Public Health (AIPH) Laboratory Science (LS) personnel. LS personnel will determine the most appropriate analytical method for analyzing the concentration of NTO in blood. The details of the analytical method will be documented in the study records and final report. Two rats will be used for each inhalation exposure and these rats will be obtained from the "additional" rats ordered from the acute inhalation test (Experiment 1) for the purpose of weight matching. A maximum of 5 exposures will be conducted, so a maximum of 10 rats will be used for

inhalation administration during Experiment 2. Additionally, 2 rats will be used for each oral gavage dose group. These rats will be ordered specifically for the oral dose administration phase of the test in which 2 rats will be receive an oral gavage dose of NTO. A maximum of 5 dose groups will be conducted (to coincide with the maximum number of inhalation exposures), so a maximum of 10 rats will be used for the oral administration during Experiment 2. Any animals not used for this rangefinding test will be transferred to another protocol or humanely euthanized per protocol guidance.

Experiment 2. Rangefinding Blood Absorption Test

Exposure Route / Exposure or Dose No. / Design Concentration	No. of Male Rats	No. of Female Rats
Inhalation / EXP#1 / 2 mg/L or TBD	1 (a)	1 (a)
Inhalation / EXP#2 / TBD	1 (a)	1 (a)
Inhalation / EXP#3 / TBD	1 (a)	1 (a)
Inhalation / EXP#4 / TBD	1 (a)	1 (a)
Inhalation / EXP#5 / TBD	1 (a)	1 (a)
Oral / DOSE#1 / TBD	1 (b)	1 (b)
Oral / DOSE#2 / TBD	1 (b)	1 (b)
Oral / DOSE#3 / TBD	1 (b)	1 (b)
Oral / DOSE#4 / TBD	1 (b)	1 (b)
Oral / DOSE#5 / TBD	1 (b)	1 (b)
	TOTAL = 5 (a,b)	**TOTAL = 5 (a,b)**

(a) Rats will be obtained from the 2 additional rats ordered for each of the acute inhalation (LC50) exposures (Experiment 1); therefore, these rats are already accounted for in the table for Experiment 1 (see above) and are not included in the TOTAL listed here; the only rats included in the TOTAL here are the rats ordered specifically for the oral dosing phase of this rangefinding test.

(b) Two rats (one male and one female) will be ordered specifically for the oral dosing phase of this rangefinding test.

V.1.3. Experiment 3: Multi-Timepoint Blood Absorption Test

A multi-timepoint blood absorption test will be conducted to determine the absorption rate of NTO into the blood of rats following the administration of the test substance by two different routes of administration (inhalation and oral gavage). Rats will be ordered from the vendor with matching ages/weights and each rat will be received with a subcutaneous femoral artery catheter in place. One group of 6 rats (3 male and 3 female) will be exposed nose-only to a single, 4-hour atmosphere of the test substance in air and another group of 6 rats (3 male and 3 female) will receive a single, oral gavage dose of the test substance. The absorption of NTO will be determined from blood samples collected from each rat at selected timepoints during the test. A single blood sample (approximately 0.15 ml) will be drawn from each rat at selected timepoints: For the rats exposed by inhalation, blood samples will be drawn at each of 7 timepoints: (1) approximately 1-2 hours prior to initiation of the inhalation exposure, (2) approximately 1 hour from initiation of the inhalation exposure, (3) approximately 2 hours from initiation of the inhalation exposure, (4) immediately following (e.g., within 30 minutes) the conclusion of the 4-hr inhalation exposure, (5) approximately 4 hours following conclusion of the inhalation exposure, (6) approximately 8 hours following conclusion of the inhalation exposure, and (7) following an overnight recovery period (approximately 18 hours). For the rats dosed by oral gavage, blood samples will be drawn at each of 6 timepoints: (1) approximately 1-2 hours prior to the dose, (2)

approximately 1 hour following the dose, (3) approximately 2 hours following the dose, (4) approximately 4 hours following the dose, (5) approximately 8 hours following the dose, and (6) following an overnight recovery period (approximately 18 hours). The blood sample collected at each timepoint will not exceed 0.2 ml and the total blood volume collected during the 24-hour blood collection period will not exceed 7.5% of the circulatory blood volume for the rats (reference 7). Blood samples will be collected from the subcutaneous femoral artery catheter of each rat utilizing the following process:

- To draw a blood sample from the catheter, one of the study personnel will gently restrain the animal while a second individual performs the actual blood collection procedure.
- The catheter plug, together with the catheter, will be pulled 1-2 inches caudally out of the skin pocket.
- While holding the junction of the plug and the polyurethane tubing with forceps, a hemostat or a second set of forceps will be used to remove the plug.
- A 1 cc syringe fitted with a 23 gauge luer stub needle adaptor will be inserted into the catheter tubing and the lumen lock solution (heparinized solution) will be withdrawn.
- The catheter will then be crimped with a cushioned hemostat.
- A clean 1 cc syringe fitted with a 23 gauge luer stub needle adaptor will be inserted into the catheter and the hemostat will be removed in order to withdraw the blood sample.
- Approximately 0.15 ml of blood will be drawn from each rat at each sample timepoint for whole blood analysis.
- After the blood samples are withdrawn, the catheter will be crimped with a hemostat and a saline-filled syringe will be inserted into the catheter.
- The catheter will be flushed with approximately 0.2 ml of saline solution and crimped again with a hemostat.
- A syringe filled with heparinized saline solution will be inserted into the catheter and the dead volume of the catheter will be filled with heparinized saline solution and plugged in order to prevent clotting.
- The catheter may be wiped with an alcohol swab if necessary, inserted back into the skin flap, and secured in place with a wound clip (reference 8).

Blood samples will immediately be injected into a vessel containing a measured volume of solvent specified by AIPH Laboratory Science (LS) personnel. LS personnel will determine the most appropriate analytical method for analyzing the concentration of NTO in blood. The details of the analytical method will be documented in the study records and final report. Following the final blood collection sample, each rat will be euthanized by injection of a solution of sodium pentobarbital into the catheter and euthanasia will be ensured by pneumothorax. A total of 14 rats will be needed to conduct this blood absorption test. One additional rat/sex will be ordered to ensure that if there are any problems with the catheter there are at least 6 rats/sex/administration route remaining to place on test. Since it is not critical to have similar body weights for the rats being tested, no attempt will be made to perform any type of computerized, randomization program for grouping. Animals not used for this blood absorption test will

not be transferred to another protocol due to the subcutaneous femoral artery catheter, and therefore, will be humanely euthanized per protocol guidance.

Experiment 3. Multi-Timepoint Blood Absorption Test

Exposure Route / Design Concentration	No. of Male Rats	No. of Female Rats
Inhalation / 2 mg/L or TBD	3	3
Oral / TBD	3	3
Additional Rats Ordered for Potential Catheter Clogging Problems	1	1
	TOTAL = 7 (a)	**TOTAL = 7 (a)**

(a) Six rats (3/sex) will be used for each route of exposure. One additional rat/sex will be ordered for weight matching purposes. However, since 2 of the rats will be obtained from the 2 additional rats ordered for one of the acute inhalation (LC50) exposures (Experiment 1), these rats are already accounted for in the table for Experiment 1 (see above). The other 2 rats will be ordered specifically for this rangefinding test.

V.1.4. Test Substance: This study will be conducted with 3-nitro-1,2,4-triazol-5-one (NTO). A sample of this test substance was supplied by BAE SYSTEMS, Ordnance Systems, Kingsport, TN for use in a previously conducted subchronic oral toxicity study in rats. The test sample was received at the test facility in November 2008 and is identified as Batch# 10NTO7-3 and Lot# BAE07B305-001. Additional sample(s) of the test substance containing particle size characteristics more compatible for inhalation toxicity testing may be received from BAE SYSTEMS. If additional samples of the test substance are received, appropriate identification documentation will be made in the study records. To facilitate generation, NTO may be mixed with an appropriate solvent (e.g., water) prior to generating it in the exposure chamber. If mixed with water, the resulting test mixture will be monitored for potential acidity concerns and appropriate buffer(s) may be added (e.g., sodium bicarbonate) to adjust the pH of the mixture such that it is maintained between 2.0 to 7.0. Relevant study guidelines (e.g., OPPTS) and TOX SOPs (e.g., TOX SOP 007) restrict the use of test substances with pH extremes (pH <2 or >11.5) for animal testing (references 9 and 10). Following is a list of relevant information concerning the chemical/physical properties of the test substance.

Test Substance Chemical/Physical Properties

Name	3-nitro-1,2,4-triazol-5-one
Synonym	NTO
CAS#	932-64-9
Physical State	White to pale yellow crystalline powder
Molecular Formula	$C_2H_2N_4O_3$
Molecular Weight	130
Density	1.93 g/cm^3
Solubility	Soluble in water (16 g/L)
Purity (by HPLC)	99.6%

V.1.5. Administration of Test Substance by Inhalation: Rats being evaluated for inhalation toxicity and blood absorption of NTO via inhalation administration will be exposed nose-only to airborne concentrations of the test substance. The nose-only (head-only) exposure mode is typically used for test atmospheres that contain particulates/aerosols in an attempt to minimize deposition of the test substance onto the fur of the animals, and therefore, minimizing inadvertent dermal and oral exposure of

the test substance to the animals. Since the generation method of NTO for this study is expected to produce an aerosol test atmosphere, the nose-only exposure mode is considered to be the most appropriate mode. Rats will be individually restrained during exposure in perforated, stainless steel cylinders with conical nose pieces. This type cylinder design is typically used for nose-only inhalation exposures and is widely accepted for inhalation toxicity test systems (references 11 and 12). Rats will be positioned in the exposure cylinders such that their noses will be at the conical end of the cylinder. In order to secure the rat in this position, a plastic disk with a hole in the center will be inserted over the tail of each rat and positioned within the cylinder near the base of the rat's tail to prevent the rat from backing out of the rear of the cylinder. Care will be taken to properly insert each rat into its exposure cylinder, such that there is a balance between allowing the rat adequate space to move while ensuring that it is positioned properly for adequate exposure. Each exposure cylinder will be inserted into one of the holes in the faceplate of the exposure chamber such that only the nose/head of each rat extends into the exposure chamber. Animal nose-only exposure cylinders and related equipment will be appropriately cleaned after each use.

V.1.6. Administration of Test Substance by Oral Gavage Dose: Rats being evaluated for blood absorption of NTO via oral administration will be orally dosed (gavaged) using a stainless steel 16 ga x 2 inch gavage needle. As per EPA Health Effects Test Guidelines, the volume given will not exceed 10 ml/kilogram of body weight (reference 13). Due to the acute nature of the blood absorption tests, no attempts will be made to analyze the purity/concentration of the NTO solutions to be dosed.

V.1.7. Concentration Selection for Inhalation Exposure(s): For the acute inhalation toxicity test (LC_{50}), the design concentration for the initial exposure will be based on the acute oral toxicity classification of NTO. NTO was determined to have an LD_{50} of greater than 2000 mg/kg in a previous study and therefore is considered to be no worse than slightly toxic by acute oral administration (reference 14). If acute inhalation toxicity of NTO is similar to the acute oral toxicity, the design concentration for the initial inhalation exposure will be conducted at the limit concentration (2 mg/L). However, due the physical properties and characteristics of the test material, the limit concentration may not be attainable, and therefore, the initial exposure may need to be conducted at the highest concentration that can reasonably be generated. Method development work conducted prior to the animal exposure(s) will help to determine the concentration levels able to be generated. If additional exposures are needed following the initial exposure, design concentrations for these subsequent exposures will be appropriately adjusted based on the results of previous exposure(s). For the blood absorption tests, the design concentration for the inhalation exposures will be based on results of the acute inhalation test and/or any rangefinding work conducted during this study in conjunction with analytical guidance from AIPH Laboratory Sciences (LS) personnel to ensure appropriate limits of detection for blood analysis.

V.1.8. Dose Selection for Oral Gavage: Dose selection for the rats administered oral doses as part of the blood absorption tests will be based on the highest-obtainable concentration reached for the inhalation exposure with NTO and the acute oral toxicity classification for NTO. The acute oral toxicity classification for NTO was determined in a previous study (reference 14) in which NTO was classified as being no worse than

slightly toxic by acute oral toxicity (LD_{50} > 2000 mg/kg). Referring to the EPA Toxicity Categories (reference 9), the concentration range listed for a test substance categorized as slightly toxic by acute oral testing ranges from 500 to 5000 mg/kg. Therefore, the oral dose administered to rats on the blood absorption tests will likely range from 500 to 5000 mg/kg. The actual dose selection will be based on results of method development work conducted during this study in conjunction with analytical guidance from AIPH Laboratory Sciences (LS) personnel to ensure appropriate limits of detection for blood analysis.

V.1.9. Inhalation Exposure Duration: For inhalation administration, each group of rats will be exposed for 4 hours to the test atmosphere. For the acute inhalation toxicity test and the rangefinding blood absorption test, the starting time of the exposure will be defined as the time when the generation system is turned on. The ending time of the exposure will be defined as the time when the generation system is turned off. Rats will be exposed to the test substance during both the time it takes for the chamber to reach concentration, and the time it takes for the test substance to be purged from the chamber. For the multi-timepoint blood absorption test, the rats will be loaded into the exposure system chamber in staggered increments (e.g., approximately 15-minute increments) in order to accommodate the blood collection schedule. The time that each rat is loaded into the exposure system will be recorded in the study records and will represent the beginning of the exposure period for that individual rat. Rats will be removed from the exposure chamber approximately one and two hours after the start of the exposure so that blood samples can be collected. The time that each rat is removed from the chamber, the time of the blood collection, and the time that each rat is returned to the exposure chamber will be recorded in the study records. The time that rats are not in the exposure chamber during the blood collections will be made up at the end of the exposure to ensure that each rat receives a 4-hour exposure. At the end of each exposure, all rats will be removed from the exposure cylinders.

V.1.10. Inhalation Atmosphere Generation: Attempts will be made to generate atmospheres of the NTO in the exposure chamber which can be readily respired by the rats being exposed and which approximate the physical form of the test substance expected to be encountered in real-life situations. Chamber atmospheres will be generated dynamically, and attempts will be made to produce evenly distributed mixtures of the test substance in air with a minimum airflow of at least 10 air changes per hour in the exposure chamber. Measurements will be taken during the method development phase and the generation system altered, if needed, to strive for uniform distribution of the test substance within the breathing zone of the exposed animals. The methods of chamber distribution are described in TOX SOP 152 (reference 15). The test substance will either be generated as a dry dust or may need to be mixed with an appropriate solvent (e.g., water) and test atmospheres will be generated by aerosolizing the test mixture. Attempts will be made to generate respirable-sized particles (e.g., mass median aerodynamic ranges from 1 to 4µm) that would be expected to be deposited throughout the respiratory tract. If the targeted particle size cannot be attained, the most respirable atmosphere practically attainable will be tested. The interior atmosphere of the exposure chamber will be slightly negative in relation to its surroundings. Test atmospheres will be exhausted through appropriate exhaust equipment (e.g., scrubbers, HEPA filters) prior to discharge into ventilated exhaust

piping. The actual generation equipment and experimental conditions used will be documented in the study records and described in the final report.

V.1.11. Analysis of the Test Atmosphere: A suitable analytical method, approved by the study director, will be used to determine the atmospheric concentration (and particle size) of the test substance in the general breathing zone of the exposed rats. The method of aerodynamic particle size measurement is described in TOX SOP 041 (reference 16). Chamber analysis samples will typically be collected by TOX study personnel, however, some analyses may need to be performed by AIPH Laboratory Sciences (LS) personnel. Unless prohibited by the nature of the test substance, chamber environmental conditions (e.g., airflow, temperature, humidity, etc.) will be monitored continuously and collected at least 3 times during each exposure. All exposure chamber analytical and environmental data will be documented in the study records. Details of the actual analytical methods and equipment used will be documented in the study records and described in the final report.

V.1.12. Grouping of Study Animals: Animals that have been released from quarantine, have no overt signs of disease, and are of the appropriate sex, age, and body weight range will be randomly selected for the study. The weight variation in animals used for the acute inhalation test exposures should not exceed ± 20% of the mean weight of each sex.

V.1.13. Animal Body Weights and Observations: All rats will be weighed at least once per week during the exposure/recovery period. Body weights will be collected (at a minimum) just prior to exposure/dosing, the day following exposure/dosing, and the final day of recovery. Additionally, rats will be weighed on the day following each instance of weight loss attributed to toxicity of the test material. Rats typically will not be weighed on weekends or holidays unless warranted by their health status. Individual body weights of animals will be documented in the study records by study personnel.

A thorough physical examination of each rat will be performed by study personnel at least once per day (weekends and holidays excluded unless warranted by health status). The examination process will consist of each rat being removed from its home cage, individually handled, and carefully observed. Observations will include, but not be limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypes or bizarre behavior (e.g., self mutilation, walking backwards). In addition, rats exposed via the inhalation route of administration will be observed at least 3 times while they are in the exposure chamber (unless restricted by the characteristics of the test atmosphere or exposure system) and will also be thoroughly observed immediately following their removal from the exposure system. Subsequent observations of rats up to several hours following the exposure may also be conducted by study personnel if warranted by health status of the rats. All data related to the observation of rats will be detailed and thoroughly documented in the study records by study personnel.

In addition, a brief summary related to the collection of body weights and observations will also be recorded in the animal room logbook on days that this data is collected. If any animals die during the exposure or recovery periods, the day and time of death will be recorded as precisely as possible. Rats will also be observed by Veterinary Medicine staff during the acclimation, exposure, and recovery periods (including weekends and holidays).

V.1.14. Gross Pathology: A gross necropsy will be performed on all animals tested as part of the acute inhalation toxicity test (Experiment 1). Following the 14-day recovery period, all surviving rats will be euthanized by carbon dioxide and undergo a gross necropsy. Based on findings during the gross necropsy, some tissues may be saved for future histopatholgical examination. The decision to save tissues will be documented in the study records and the results will be included in the final report. All data related to the necropsy will be recorded on CHPPM Form 333. If the necropsy cannot be performed immediately after the death of an animal, the animal will be refrigerated at temperatures low enough to minimize autolysis.

V.1.15. Study Conduct: This study will be conducted in a manner consistent with the principles of 40 CFR (Code of Federal Regulations) Part 792 "Toxic Substance Control Act" (TSCA) Good Laboratory Practice (GLP) Regulation (reference 17). All study records will be made available to oversight organizations such as the Environmental Protection Agency or the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) as needed. The investigators and technicians will adhere to The Guide for Care and Use of Laboratory Animals, 2011 (reference 18).

Records will be kept in standard USAPHC laboratory notebooks and/or three ring binders. Daily records will be kept on survival and clinical signs collected on the animals during the exposure and recovery periods. Procedures for preparation of any euthanasia solution, drug administration, animal bleeds, observation logs, morbidity/mortality logs, etc., will be stored with the study records. These records will be made available to oversight organizations such as the US EPA, AAALAC, and the IACUC. The protocol, protocol amendments, raw data, statistical analysis, tabular calculations, and graphic analysis of the data will be saved with the study records. Additionally, memoranda to the study file, study logs, signature logs, final reports, and final report amendments will be archived at USAPHC. Some ancillary records such as maintenance and calibration logs, environmental monitoring logs, animal room log books, all veterinarian staff duties logbooks, training files, etc. may be stored in the archives but not stored with the study files.

V.2. Data Analysis: For the acute inhalation toxicity test, if the test substance fails the limit concentration test, then an LC_{50} value will be estimated using a probit analysis (reference 19) and 95% confidence limits estimated if possible. In the absence of confidence limits, an approximate LC_{50} may be reported. For the multi-timepoint blood absorption test, a comparison of the concentration of NTO in the blood samples across times will be performed with a randomized block analysis of variance (ANOVA) followed by a Tukey's or Dunnett's C test to compare pairs of times. The use of 6 animals per dose administration is considered adequate for determination of a 50% or greater difference in test material blood concentrations between groups at alpha equal 5% with

power of 80%. Statistical significance for all tests is defined as $p<.05$. Descriptive statistics (e.g., mean, standard deviation, and standard error of the mean) will be used to summarize experimental data (e.g., atmospheric concentrations). If additional statistics are required, they will be documented in the study records and included in the final report.

V.3. Laboratory Animals Required and Justification

V.3.1. Non-animal Alternatives Considered: The objective(s) addressed by this study are adverse health effects observed in rats administered the test substance by inhalation and oral administration. The data from this study will aid in the assessment and evaluation of the toxic characteristics of the test substance. There are no appropriate animal substitutes (e.g., computer models, tissue/cell cultures) for the data that will be produced in this study. No non-animal alternative would provide the necessary toxicological information provided by this study. Therefore, it is necessary to perform this study in an animal model.

V.3.2. Animal Model and Species Justification: The test guidelines for the U.S. Environmental Protection Agency (EPA) and Organisation for Economic Co-Operation and Development (OECD) state that the rat is the preferred species (references 20 and 21). Sprague-Dawley rats are the strain of rat that have been historically used for acute inhalation and oral toxicity studies by USAPHC TOX and are the recommended species due to an historical and extensive database.

V.3.3. Laboratory animals

V.3.3.1. Genus and Species: *Rattus norvegicus*

V.3.3.2. Strain/Stock: Sprague-Dawley

V.3.3.3. Source vendor: Charles River Laboratories, Wilmington, MA (USDA 14-R-0144) or other USAPHC approved vendor

V.3.3.4. Age (at exposure): Approximately 8-10 weeks

V.3.3.5. Weight (at exposure): Age appropriate

V.3.3.6. Sex: Male and female. Only one sex (the more sensitive sex) will be used when obvious sex differences relative to toxicity are noted.

V.3.3.7. Special Considerations: None

V.3.4. Number of Animals Required (By Species):

Minimum of 28 rats (if only one acute inhalation toxicity exposure is conducted)

NUMBER OF RATS IF MINIMUM NUMBER OF EXPOSURES CONDUCTED

Type Test	Concentration	# Rats Ordered	# Rats Exposed/Dosed	# Additional Rats
Acute Inhalation (LC_{50})	2000 mg/m^3	12	10	2
Rangefinding Blood Absorption	TBD	2	4	(a)
Multi-Timepoint Blood Absorption	TBD	14 (b)	12	2
TOTAL		**28**	**26**	**2 (a)**

(a) Two rats (one/sex) will be used for each route of exposure (total of 4 rats). Two of the rats will be obtained from the 2 additional rats ordered for the acute inhalation (LC50) exposure and the other 2 rats will be ordered specifically for this rangefinding test.
(b) Each rat will be received from the animal supplier with a subcutaneous femoral artery catheter in place

Maximum of 84 rats (if a total of 5 acute inhalation toxicity exposures are conducted)

NUMBER OF RATS IF MAXIMUM NUMBER OF EXPOSURES CONDUCTED

Type Test / Exposure No.	Concentration	# Rats Ordered	# Rats Exposed/Dosed	# Additional Rats
Acute Inhalation (LC_{50}) / Exp#1	2000 mg/m^3	12	10 *[Pain Category C]*	2
Acute Inhalation (LC_{50}) / Exp#2	TBD	12	10 *[Pain Category C]*	2
Acute Inhalation (LC_{50}) / Exp#3	TBD	12	10 *[Pain Category C]*	2
Acute Inhalation (LC_{50}) / Exp#4	TBD	12	10 *[Pain Category E]*	2
Acute Inhalation (LC_{50}) / Exp#5	TBD	12	10 *[Pain Categor E]*	2
Rangefinding Blood Absorption	TBD	10 (a)	20 (a) *[12 rats @ Pain Cat D] [8 rats @ Pain Cat E]*	0 (a)
Multi-Timepoint Blood Absorption	TBD	14 (b)	12 (b) *[Pain Category C]*	2 (b) *[Pain Category B]*
TOTAL		**84**	**82**	**2 (a)**

(a) Two rats (one/sex) will be used for each inhalation exposure (total of 10 rats); these 10 the rats will be obtained from the 2 additional rats ordered for each of the acute inhalation (LC50) exposures; two rats (one/sex will be used for each oral gavage dose (total of 10 rats); these 10 rats will be ordered specifically for the rangefinding test.
(b) Each rat will be received from the animal supplier with a subcutaneous femoral artery catheter in place

For the acute inhalation toxicity test (LC_{50}), at least one group of 10 rats will be exposed to a design concentration of 2000 mg/m^3 NTO (if attainable), however, depending on the results of this exposure and any subsequent exposures, as many as 4 additional exposures may need to be conducted to accurately determine the inhalation median

lethal concentration (LC_{50}) for NTO. Twelve rats (6 male and 6 female) will be ordered for each of the acute inhalation toxicity (LC_{50}) exposures, with 10 rats (5 male and 5 female) being exposed during each exposure and one rat per sex designated as "additional". A minimum of 5 rats per sex for each of the acute inhalation toxicity (LC_{50}) exposures is required by the EPA and OECD Health Effects Test Guidelines (reference 20 and 21). "Additional" rats need to be ordered for weight matching purposes because these same guidelines require that the body weight variation for each exposure group of rats prior to exposure be within ± 20% of the mean weight of each sex. The "additional" animals (one female rat and one male rat) from Experiment 1 will be used for the inhalation exposure phase of the rangefinding test (Experiment 2).

Although there are no specific regulatory guidelines for the blood absorption tests to be conducted as part of this study, the number of rats being used for these tests is considered to be appropriate. For the multi-timepoint blood absorption test, a total of 7 rats/sex will be received for each dose administration (inhalation and oral), with 6 rats (3 male and 3 female) being exposed/dosed and one rat being designated as "additional". The use of 6 animals per dose administration is considered adequate for determination of a 50% or greater difference in test material blood concentrations between the two different dose administrations at alpha equal 5% with power of 80%. For the rangefinding blood absorption test, groups of 2 rats (one rat/sex) for each of the dose administrations (inhalation and oral) is considered to be the minimum number of rats required to provide adequate rangefinding results.

V.3.5. Refinement, Reduction, Replacement

V.3.5.1. Refinement: Standard rat enrichment will be implemented in accordance with TOX SOP 122 (reference 22). For rats assigned to the acute inhalation test and the rangefinding blood absorption test, rats may be pair-housed (sexes separate) during the acclimatization period. However, during the conduct of the study, rats from these tests will need to be single-housed due to the unknown toxicity of the test substance. For rats assigned to the multi-timepoint blood absorption test, rats will need to be singly housed upon their arrival and throughout the test due to the subcutaneous femoral artery catheter that each of the rats will have in place upon arrival. All animals on this study will be handled on a frequent basis and provided a form of environmental enrichment (e.g., nylabones) throughout the study period, except during the 4-hour exposure period. Another refinement is that moribund animals or animals in overt pain and unlikely to recover will be humanely euthanized.

V.3.5.2. Reduction: Ten rats (e.g., 5 male and 5 female) per exposure is the number of animals specified by the applicable regulatory guidelines for an acute inhalation toxicity study (references 20 and 21). Although there are no specific regulatory guidelines for blood absorption tests being conducted as part of this study, the number of animals being requested is in the range of generally accepted number per sex for standard acute inhalation/oral toxicity studies. No control groups will be used. Tissue sharing may be allowed (except for rats with subcutaneous femoral artery catheter), however, only if doing so will not affect the validity of the study.

V.3.5.3. Replacement: No non-animal alternatives are known to exist that will provide the required data. At this time, there are no non-animal alternatives that can fully replicate the complex processes that occur within an intact mammalian organism.

V.4. Technical Methods

V.4.1. Pain/Distress Assessment:

V.4.1.1. APHIS Form 7023 Information

V.4.1.1.1. Number of Animals
NOTE: Estimates listed in Columns B-E below are modeled after a maximum number of 5 exposures conducted for the acute inhalation toxicity test.

V.4.1.1.1.1. Column B: 2 rats (2 "additional" rats ordered with subcutaneous femoral artery catheter for use on the multi-timepoint blood absorption test)

V.4.1.1.1.2. Column C: 42 rats (10 rats per exposure at 3 lower concentrations conducted for the acute inhalation test; 12 rats ordered with subcutaneous femoral artery catheters to be exposed/dosed as part of the multi-timepoint blood absorption test)

V.4.1.1.1.3. Column D: 12 rats (2 rats per exposure at the 3 lower concentrations for the inhalation phase of the rangefinding test; 2 rats per dosing concentration at the 3 lower concentrations for the oral dosing phase of the rangefinding test)

V.4.1.1.1.4. Column E: 28 rats (10 rats per exposure at 2 higher concentrations conducted for the acute inhalation test; 2 rats per exposure at the 2 higher concentrations for the inhalation phase of the rangefinding test; 2 rats per dosing concentration at the 2 higher concentrations for the oral dosing phase of the rangefinding test)

V.4.1.2. Pain Relief/Prevention

V.4.1.2.1. Anesthesia/Analgesia/Tranquilization: For the rats assigned to the rangefinding blood absorption test, anesthesia will be administered prior to cardiac blood collection and euthanasia. Anesthesia will consist of isoflurane or CO_2 gas. For isoflurane anesthesia, study staff will ensure the oxygen tank and isoflurane levels are sufficiently full and scavenger canisters are connected to both exhaust lines. The stopcock to the box will be turned to the open position and the stopcock to the nosecone to the off position. The oxygen tank will be turned on, the flow meter set to 1 L/min, the rat placed in the plastic box, and the lock latched. The isoflurane valve will be turned to approximately 3%. Once the rat is sufficiently anesthetized (immobile and not responsive to tapping on the box), the stopcock to the nosecone will be switched to on and the stopcock to the box to off. The rat will be transferred to the nosecone and it will be ensured that the rat is still sufficiently anesthetized, based on lack of responsiveness to toe-pinch, before performing terminal blood sampling. For CO_2 anesthesia, study staff will ensure that the CO_2 tank is sufficiently full and connected to

the CO_2 chamber. The rat will be placed in the CO_2 chamber, the lid put on the chamber, and the CO_2 valve turned on at a low flow (approx, ¼ turn on the tank valve). When the rat is sufficiently anesthetized (shallow breathing pattern) it will be removed from the chamber and immediately placed on a necropsy board, where prior to performing the terminal blood sampling, sufficient anesthetization will be ensured by the rat's lack of responsiveness to a toe-pinch.

V.4.1.2.2. Pre- and Post-procedural Provisions: Animals will be monitored just prior to exposure/dosing, during exposures, and immediately following exposure (while being returned to their cages) and/or dosing. A careful clinical examination will be made at least once each day during the observation period. Appropriate actions will be taken to minimize loss of animals to the study or associated relevant data (e.g., necropsy or refrigeration of those animals found dead). Observations will be detailed and carefully recorded in the study records. Observations will include, but not be limited to, evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypes or bizarre behavior (e.g., self mutilation, walking backwards). Observation and body weight frequency is described in detail in section V.2.12.

V.4.1.2.3. Paralytics: None

V.4.1.3. Literature Search for Alternatives to Painful or Distressful Procedures

V.4.1.3.1. Source(s) Searched: AGRICOLA, FEDRIP, NTIS

V.4.1.3.2. Date of Search: 08 March 2011

V.4.1.3.3. Period of Search: 1964-2011

V.4.1.3.4. Key Words of Search: 3-nitro-1,2,4-triazol-5-one, nitro compounds, triazoles, nto, aerosol, inhalation, breath, lung, pulmonary, respiration, toxicity, blood, concentration, alternative, welfare, method, model, in vitro, pain, distress, simulate, video, computer, replacement, refinement, reduction

V.4.1.3.5. Results of Search: The literature search identified 16 references pertaining to alternatives to painful procedures. However, no alternatives to the painful or distressful procedures (e.g., illness resulting from administration of the test substance, cardiac bleed) in this protocol or methods to relieve pain or distress without altering the outcome of the study were found. Since the goal of this investigation is to determine the effects from acute inhalation and/or oral exposure (e.g., lethality from a one-time administration of the test substance) the observation of illness associated with toxicity is necessary. However, moribund animals or animals in overt pain unlikely to recover will be humanely euthanized as described in section V.4.6. Because no validated in vitro tests are currently available to replace in vivo inhalation/oral toxicity studies, this protocol must be conducted in vivo, necessitating possible painful procedures or illness.

V.4.1.4. Unalleviated Painful/Distressful Procedure Justification: The nature of this type of study precludes the use of totally painless procedures. Since the objective of this study is to determine the toxicological effects from acute inhalation exposure and/or acute oral dosage to the test substance, no materials that could potentially interfere or mask the interpretation of these toxicological effects will be administered to the study animals. The primary endpoint of the acute inhalation test is death, therefore, it is important that the investigators are confident in the outcome before pain or distress can be alleviated. Since animals will receive a single dose of the test substance, subsequent recovery may occur. Therefore, pain and/or distress will be alleviated only if it is judged that animals are unlikely to recover. Administration of anesthetics, analgesics, or drugs in this model to alleviate pain or distress is untested and may alter the manifestation of the toxic response to the compound, and thus compromise the results of the experiment. Typical pain relievers such as opiates and non-steroidal anti-inflammatories as well as anesthetics have the ability to mask certain toxic signs that may be observed due to the administration of the test compound, especially those signs resulting from pain or distress. In addition, certain side effects such as alterations in blood chemistry and hematology may arise from the use of these drugs and could be misinterpreted by the investigator as clinical signs caused by the test material. In addition, the observation of the onset, duration and/or reversibility of toxic signs is critical to mechanistic interpretation; "Toxic signs" are defined in TOX SOP 063 (reference 23). However, the Attending Veterinarian will be consulted to evaluate animals that appear moribund and the Attending Veterinarian and Primary Investigator/Study Director (PI/SD) will determine if euthanasia is indicated for these animals. One or more of these clinical signs will be considered to be indicative of a moribund animal: impaired ambulation which prevents animals from reaching food/water; excessive weight loss or emaciation (≥ 20% body weight loss from start of test); lack of physical or mental alertness; prolonged labored breathing; unabated seizure activity; inability to urinate or defecate for greater than 24 hours; or a prolonged inability to remain upright. Animals considered to be moribund will be euthanized as described in section V.4.6. The final number of rats in each pain category will be reported to the IACUC annually and at the completion of the in-life portion of the protocol.

V.4.2. Prolonged Restraint: For nose-only exposures, rats will be restrained in perforated, stainless steel cylinders during the 4-hour exposure period. This type of restrainer and the restraint regimen is a commonly accepted method of restraint for rats used during nose-only inhalation exposures (references 11 and 12). Rats will be contained within the nose-only cylinders longer than the 4-hour exposure period due to the additional time required to load the rats into the cylinders prior to the exposure and then unloading them from the cylinders following the exposure. The time period that the rats are actually contained within the exposure cylinder, allowing for the loading/unloading process, however, will not exceed 5 hours. Current IACUC policy defines prolonged restraint as any restraint greater in duration than 15 minutes and requires that restrained animals be habituated or undergo acclimation to the restraint device in the event of prolonged restraint unless scientifically justified to be unnecessary (reference 24). Acclimation of the rats to the nose-only exposure cylinders is not considered to be scientifically justified for this study because it is a single exposure and the primary endpoint is mortality. If the endpoints for this study included collection of

critical parameters such as sensitive blood chemistries or physiological measures, or if it was a repeated-dose study evaluating endpoints such as body weights, acclimation of the rats to the exposure cylinders would probably be considered scientifically justified. However, since this is a single-dose study, it is not considered necessary to acclimate the animals on this study to the nose-only exposure cylinders prior to exposure. Furthermore, the exposure cylinders are not considered to be stressful to the rats in and of themselves since their design is similar to the enrichment tubes typically placed in the rats' cages. The only stressful situation for the rats restrained in these exposure cylinders is considered to be the first few minutes of exposure to the test atmosphere, and an acclimation period to the exposure cylinder prior to the actual exposure would not prevent this type of stress.

V.4.3. Surgery: None

V.4.3.1. Pre-Surgical Provisions: N/A

V.4.3.2. Procedure: N/A

V.4.3.3. Post-Surgical Provisions: N/A

V.4.3.4. Location: N/A

V.4.3.5. Surgeon: N/A

V.4.3.6. Multiple Major Survival Operative Procedures: None

V.4.3.6.1. Procedures: N/A

V.4.3.6.2. Scientific Justification: N/A

V.4.4. Animal Manipulations

V.4.4.1. Injections: None

V.4.4.2. Biosamples: For the rats assigned to the rangefinding blood absorption test, approximately 3-6 ml of blood will be taken from each rat just prior to euthanasia. All blood sampling will occur under isoflurane or CO_2 gas anesthesia via cardiac puncture using an 18-21 gauge, 1-1.5 inch needle, as outlined in TOX SOP 053 (reference 6). Biosampling will be promptly followed by euthanasia via CO_2. For the 12 rats assigned to the multi-timepoint blood absorption test, a single blood sample (approximately 0.15 ml) will be drawn from the subcutaneous femoral artery catheter of each rat at each of up to 7 timepoints (see section V.2.3 for details). The blood sample collected at each timepoint will not exceed 0.2 ml and the total blood volume collected during the 24-hour blood collection period will not exceed 7.5% of the circulatory blood volume for the rats (reference 7). Following the final blood collection sample, each rat will be euthanized by injection of approximately 1 ml of a solution of sodium pentobarbital into the catheter or by exposure to CO_2.

V.4.4.3. Adjuvants: N/A

V.4.4.4. Monoclonal Antibody (MAbs) Production: N/A

V.4.4.5. Animal Identification: Animals will be identified by cage cards according to TOX SOP 003 (reference 25). An identification number (e.g., the last 3 digits of the animal number) will also be marked on the tail of each rat with a water-insoluble marker in order to ensure proper identification of rats when removed from their cages or inhalation exposure system.

V.4.4.6. Behavioral Studies: N/A

V.4.4.7. Other Procedures: N/A

V.4.4.8. Tissue Sharing: Tissue sharing may be allowed upon request provided there is no affect on the validity of the study. Rats fitted with a subcutaneous femoral artery catheter will not be available for tissue sharing.

V.4.5. Study Endpoint: The study endpoint is mortality, intervention euthanasia of moribund animals, or euthanasia. The duration of the recovery period will not typically exceed 14 days. In the event that significant signs of toxicity (e.g., mortality, neurotoxicity, etc.) are delayed, the duration of the recovery period may be extended in order to determine the length of time for recovery, however, the recovery period will not exceed 28 days. The possibility exists that a compound-related death may occur during an unobserved period (i.e., overnight). Intervention euthanasia will be conducted on moribund animals. Animals will be assessed for moribundity based on a weight of evidence of the following signs: impaired ambulation which prevents animals from reaching food/water; excessive weight loss or emaciation (≥ 20% body weight loss from start of test); lack of physical or mental alertness; prolonged labored breathing; unabated seizure activity; inability to urinate or defecate for greater than 24 hours; or a prolonged inability to remain upright. Any animal considered moribund will be humanely euthanized as described in section V.4.6. The Attending Veterinarian will be consulted, if needed, to evaluate potentially moribund animals, unless the PI/SD plans to immediately euthanize the animal. The time at which signs of toxicity appear, their duration, and the time to death are important, especially if there is a tendency for deaths or morbidity to be delayed or if the signs of toxicity are reversible or recovery is possible. As such, potentially moribund animals will be monitored, in consultation with the Attending Veterinarian, for possible reversal and recovery of toxic signs.

At the end of the recovery period, all surviving animals assigned to the acute inhalation test and the rangefinding blood absorption test will be euthanized by CO_2 ensured by pneumothorax. Rats assigned to the multi-timepoint blood absorption test will be euthanized by injection of a solution of sodium pentobarbital into their subcutaneous femoral artery catheter.

V.4.6. Euthanasia: Euthanasia of rats assigned to the acute inhalation toxicity test and the rangefinding blood absorption test will be accomplished by asphyxiation from CO_2 exposure according to TOX SOP 066 (reference 26). Rats assigned to the multi-

timepoint blood absorption test, may be euthanized by injection of a solution of sodium pentobarbital (approximately 1 ml) into their subcutaneous femoral artery catheter (however, rats may also be euthanized by CO_2 exposure). Death of all rats will be ensured with a thoracotomy. Study staff will euthanize the animals.

V.5. Veterinary Care

V.5.1. Husbandry Considerations: The animals will be housed in plastic, solid-bottom shoebox cages and given water and certified rodent feed *ad libitum* during the study. For rats assigned to the acute inhalation test and the rangefinding blood absorption test, rats may be pair-housed (sexes separate) during the acclimatization period. However, during the conduct of the study, rats from these tests will need to be single-housed due to the unknown toxicity of the test substance. For rats assigned to the multi-timepoint blood absorption test, rats will need to be singly housed upon their arrival and throughout the test due to the subcutaneous femoral artery catheter that each of the rats will have in place upon arrival. Animal rooms will be maintained according to the conditions specified in TOX SOP 004 (reference 27). For rats assigned to the acute inhalation test and the rangefinding blood absorption test, animals will undergo an acclimation period of no less than 5 days after their arrival in the animal facility. Rats assigned to the multi-timepoint blood absorption test will not have the standard 4- to 5-day acclimatization period. Instead, these rats will be exposed/dosed the day following arrival at the testing facility to minimize the potential for clogging of the subcutaneous femoral artery catheter that could occur with a longer acclimatization period.

V.5.1.1. Study Room: Studies will be conducted at the USAPHC Toxicology Portfolio animal facility, Bldg E-2100 or Bldg E-2101, study room as assigned. The animal facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

V.5.1.2. Special Husbandry Provisions: Rats assigned to the acute inhalation test and the rangefinding blood absorption test may be pair-housed (sexes separate) during the acclimatization period. However, during the conduct of the study, rats from these tests will need to be single-housed due to the unknown toxicity of the test substance. For rats assigned to the multi-timepoint blood absorption test, due to the subcutaneous femoral artery catheter that each of the rats will have in place, these rats will need to be singly housed upon their arrival and throughout the test.

V.5.1.3. Exceptions: For the acute inhalation toxicity test and the rangefinding blood absorption test, body weight and observation data may also be collected for rats by study personnel during the acclimation period in an attempt to more accurately monitor the health status of the rats in preparation for their use on study. However, animals will not be weighed or handled by study personnel within the first 24 hours after their arrival to the facility. For the multi-timepoint blood absorption test, since these rats will be fitted with subcutaneous femoral artery catheters, the catheters may be flushed by study personnel on the day of their arrival in an attempt to minimize plugging of the catheter.

V.5.2. Veterinary Medical Care

V.5.2.1. Routine Veterinary Medical Care: All animals will be observed daily by assigned Veterinary Medicine personnel for husbandry conditions, humane care, and general health. Animals will be observed at least twice daily by assigned Veterinary Medicine personnel (once daily on weekends and holidays). During the exposure period, animals exposed by inhalation will be observed by study personnel prior to loading them into the exposure chamber, during the exposure, and then again after they are removed from the exposure chamber. In addition, during the recovery period, study animals will be observed at least once daily (weekends and holidays excluded) by study personnel. Observations will include, but not be limited to: evaluation of skin and fur, eyes and mucous membranes, respiratory and circulatory effects, autonomic effects such as salivation, central nervous system effects, including tremors and convulsions, changes in the level of activity, gait and posture, reactivity to handling or sensory stimuli, altered strength, and stereotypes or bizarre behavior (e.g., self mutilation, walking backwards). If there is a need for increased frequency of observations, the duty veterinarian will consult with the PI/SD. Appropriate actions will be taken to minimize loss of animals to the study (e.g., necropsy or refrigeration of those animals found dead). If the observed toxicity indicates a need for more frequent observations, the Attending Veterinarian will consult with the PI/SD.

All rats will be weighed at least once per week during the exposure/recovery period. Body weights will be collected (at a minimum) just prior to exposure/dosing, the day following exposure/dosing, and the final day of recovery. Additionally, rats will be weighed on the day following each instance of weight loss attributed to toxicity of the test material. Rats typically will not be weighed on weekends or holidays unless warranted by their health status.

Observation and body weight data collected by study personnel will be documented in the study records. A brief summary related to the collection of body weights and observations will also be recorded in the animal room logbook on days that this data is collected.

V.5.2.2. Emergency Veterinary Medical Care: All emergency animal health care will be provided by the Veterinary Medicine staff in consultation with the PI or designee whenever possible.

V.5.3. Environmental Enrichment

V.5.3.1 Enrichment Strategy: All enrichment will be provided in accordance with TOX SOP 122 (reference 22). Animals will be handled on a frequent basis and provided a form of environmental enrichment (e.g., nylabones) throughout the study, except during the 4-hour exposure period.

V.5.3.2. Enrichment Restriction: For rats assigned to the acute inhalation test and the rangefinding blood absorption test, rats may be pair-housed (sexes separate) during the acclimatization period. However, during the conduct of the study, rats from these tests will need to be single-housed due to the unknown toxicity of the test substance. For rats assigned to the multi-timepoint blood absorption test, due to the subcutaneous

femoral artery catheter that each of the rats will have in place, these rats will need to be singly housed upon their arrival and throughout the test.

VI. STUDY PERSONNEL QUALIFICATIONS AND TRAINING:

Staff Member	Procedure	Training	Experience	Qualifications
Art O'Neill	Inhalation exposure, observations, handling, CO_2 euthanasia, necropsy	Inhalation testing experience (memo from DuPont dated Oct 2008); necropsy (Dec 2007)	30+ Yrs Animal Research	B.S., Biology; LATG
Lee Crouse	Inhalation exposure, oral gavage, observations, handling, bleeding, CO_2 euthanasia, anesthesia, necropsy	Humane Care & Use of Lab Animals (May 2000); Rodent Handling Techniques, WRAIR (includes oral gavage in rats; Nov 1996); Rat handling, gavage, injections, blood collection (July 2007); Rat cardiac bleeding under isoflurane (Dec 2008, May 2009); necropsy (Oct/Dec 2007)	16+ Yrs Animal Research	M.S., Environmental Science
Emily Lent	Oral gavage, observations, handling, bleeding, CO_2 euthanasia, Necropsy	Rat handling, gavage, injections, blood collection (July 2007); Rat bleeding techniques & tissue collection (Apr 2008); necropsy (Jul/Oct 2007, Apr 2008); Rat oral gavage (March 2008); Oral gavage in rats (May 2009)	11+ Yrs Animal Research	M.S., Wildlife Biology; Ph.D., Natural Resources and Environmental Studies
Mark Way	Observations, handling, CO_2 euthanasia, Necropsy	Rodent & Small Animal Handling workshops (2003, 2007); necropsy (May 2007)	17+ Yrs Animal Research	B.S., Biology; LAT
Terry Hanna	Observations, handling, CO_2 euthanasia, necropsy, functional observation battery (FOB)	Rodent Handling & Techniques (1992); Rodent & Small Animal Handling Workshop (2004, 2005, 2006); Rat handling and gavage (2007), rat euthanasia via CO_2 with thoracotomy (3/2009); rat isoflurane anesthesia, cardiac blood draw, & CO_2 euthanasia (2009); necropsy (2009, 2010); Functional observation battery (FOB) training (5/2007, 8/2008, 1/2009); Acoustic Startle Response (handheld clicker & startle	15+ Yrs Animal Research	ALAT

		chamber operations) (1/2009)		
Will McCain	Observations, handling, necropsy	Animal Care & Use Training (Mar 1995); Humane Care & Use of Lab Animals (May 2000); necropsy (Dec 2007, Feb/Dec 2008, Feb 2009)	30+ Yrs Animal Research	Ph.D., Toxicology
Alicia Shiflett	Observations, handling, necropsy	Rodent handling & techniques training; observations, handling/restraint, weighing, basic bleeding (Nov 2008); rat CO_2 euthanasia with thoracotomy (Mar 2009); rat necropsy & tissue collection (Mar 2008, Jan 2010)	2+ Yrs Animal Research	Associates Degree, Histology/Science

VII. BIOHAZARD/SAFETY:

In accordance with PHC Reg. 385-1, CHPPM Reg. 385-5, and TOX SOP 083, standard laboratory protection (e.g., glasses, gloves, labcoat) shall be used when handling the neat test substance. The test substance shall be stored in a sealed container at room temperature when not in use. The test substance will be handled in a laboratory fume hood when necessary. Although the precise toxicity of the test substance may not be known, information regarding its chemical family will be provided so that a reasonable assessment of its safety can be made (references 28, 29, and 30). Due to the potentially flammable/explosive properties of this material, equipment in the exposure system will be grounded.

VIII. ENCLOSURES:

A. References

IX. ASSURANCES:

IX.1. As the Study Director/ Principal Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Duplication of Effort: I have made every effort to ensure that this protocol is not an unnecessary duplication of previous experiments.

C. Statistical Assurance: I assure that I have consulted with a qualified individual who evaluated the experimental design with respect to the statistical analysis, and that the minimum number of animals needed for scientific validity will be used.

D. Biohazard/Safety: I have taken into consideration, and I have made the proper coordinations regarding all applicable rules and regulations regarding radiation protection, biosafety, recombinant issues, and so forth, in the preparation of this protocol.

E. Training : I verify that the personnel performing the animal procedures/manipulations/ observations described in this protocol are technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

F. Responsibility: I acknowledge the inherent moral, ethical, and administrative obligations associated with the performance of this animal use protocol, and I assure that all individuals associated with this project will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

G. Scientific Review: This proposed animal use protocol has received appropriate peer scientific review and is consistent with good scientific research practice.

H. Painful Procedures: I am conducting biomedical experiments which may potentially cause more than ~~momentary or slight~~ pain or distress to animals. This potential pain and/or distress (WILL) or (WILL NOT) be relieved with the use of anesthetics, analgesics and/or ~~tranquilizers.~~ I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

Arthur J. O'Neill – Study Director (PI)

20110714
Date (YYYYMMDD)

IX.2. As the Primary Co-Investigator on this protocol, I acknowledge my responsibilities and provide assurances for the following:

A. Animal Use: The animals authorized for use in this protocol will be used only in the activities and in the manner described herein, unless a modification is specifically approved by the IACUC prior to its implementation.

B. Authority: I understand that, as the Primary Co-Investigator, I am authorized and responsible for performing all procedures and manipulations as assigned to the SD/PI in the SD/PI's absence. This includes euthanasia of distressed animals.

C. Training: I verify that I am technically competent and have been properly trained to ensure that no unnecessary pain or distress will be caused to the animals as a result of the procedures/manipulations.

D. Responsibility: I acknowledge the inherent moral, ethical, and administrative obligations associated with the performance of this animal use protocol, and I assure that I will demonstrate a concern for the health, comfort, welfare, and well-being of the research animals. Additionally, I pledge to conduct this study in the spirit of the fourth "R", namely "Responsibility," which the DOD has embraced for implementing animal use alternatives where feasible and conducting humane and lawful research.

E. Painful Pro cedures: I am conducting biomedical experiments which may potentially cause more than momentary or slight pain or distress to animals. This potential pain and/or distress (WILL) or (WILL NOT) be relieved with the use of anesthetics, analgesics and/or tranquilizers. I have considered alternatives to such procedures; however, I have determined that alternative procedures are not available to accomplish the objectives of this proposed experiment.

Lee C.B. Crouse – Primary Co-Investigator

20110714
Date (YYYYMMDD)

APPENDIX A

REFERENCES

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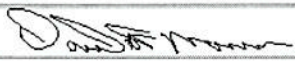
PROTOCOL REVIEW, SUPPORT, APPROVAL SHEET

PROTOCOL NUMBER:	TITLE: Acute Inhalation Toxicity and Blood Absorption of 3-Nitro-1,2,4-Triazol-5-One (NTO) in Rats
0ENT - 24 - 11-07-04 SUB-JONO TEST TYPE IACUC NUMBER	

1. SCIENTIFIC MERIT (PEER REVIEW)			
1a. Printed Name (First, MI, Last) Craig A. McFarland	1b. Title Toxicologist, HERP	1c. Signature MCFARLAND.CRAIG.A.1284367789	1d. Date (yyyy/mm/dd) 20110526
2. DIRECTOR			
2a. Printed Name (First, MI, Last) LTC Cindy A. Landgren	2b. Title Director, Toxicology	2c. Signature LANDGREN.CINDY.ANNE.1163891359	2d. Date (yyyy/mm/dd) 20110526
3. PROGRAM MANAGER			
3a. Printed Name (First, MI, Last) Glenn J. Leach	3b. Title Program Manager, TEP	3c. Signature 	3d. Date (yyyy/mm/dd) 20110524
4. ATTENDING VETERINARIAN			
4a. Printed Name (First, MI, Last) MAJ Dawn C. Fitzhugh	4b. Title Command Animal Program Manager	4c. Signature FITZHUGH.DAWN.CATHERINE.1036926127	4d. Date (yyyy/mm/dd) 20110725
5. ANALYTICAL CHEMISTRY (If Applicable)			
5a. Printed Name (First, MI, Last) David F. Morrow	5b. Title Chief, Laboratory Consultants Division	5c. Signature 	5d. Date (yyyy/mm/dd) 20110526
6. SAFETY MANAGER			
6a. Printed Name (First, MI, Last) Roy A. Valiant	6b. Title Safety Manager	6c. Signature VALIANT.ROY.A.1081780591	6d. Date (yyyy/mm/dd) 20110718
7. STATISTICIAN (If Applicable)			
7a. Printed Name (First, MI, Last) Karen D. Deaver	7b. Title Statistician	7c. Signature DEAVER.KAREN.DEVILBISS.1400519672	7d. Date (yyyy/mm/dd) 20110526

PROTOCOL NUMBER: 0ENT - 24 - 11-07-04 (SUB-JONO / TEST TYPE / IACUC NUMBER)	TITLE: Acute Inhalation Toxicity and Blood Absorption of 3-Nitro-1,2,4-Triazol-5-One (NTO) in Rats		
8. SIO-QAT (GLP COMPLIANCE AND QA SUPPORT)			
8a. Printed Name (First, MI, Last) Michael P. Kefauver	8b. Title Quality Assurance Specialist, USAPHC Quality Systems Office (QSO)	8c. Signature KEFAUVER.MICHAEL.P.1229209678	8d. Date (yyyy/mm/dd) 20110526
9. CHAIRMAN, IACUC			
9a. Printed Name (First, MI, Last) Kristin T. Newkirk	9b. Title Chairperson, IACUC	9c. Signature NEWKIRK.KRISTIN.TORELL.1014786895	9d. Date (yyyy/mm/dd) 20110729
10. INSTITUTIONAL OFFICIAL			
10a. Printed Name (First, MI, Last) John J. Resta	10b. Title Director, AIPH	10c. Signature RESTA.JOHN.J.1229129305	10d. Date (yyyy/mm/dd) 20110729
11. STUDY DIRECTOR/PRINCIPAL INVESTIGATOR			
11a. Printed Name (First, MI, Last) Arthur J. O'Neill	11b. Title Biologist, TEP	11c. Signature ONEILL.ARTHUR.J.III.1299508443	11d. Date (yyyy/mm/dd) 20110729
12. OTHER ORGANIZATION(S) PROVIDING SUPPORT (AS NEEDED):			
12a. Printed Name (First, MI, Last)	12b. Title	12c. Signature	12d. Date (yyyy/mm/dd)
13. STUDY SPONSOR:			
13a. Printed Name (First, MI, Last) Mark S. Johnson	13b. Title Program Manager, HERP (for RDECOM)	13c. Signature	13d. Date (yyyy/mm/dd)

USACHPPM PROTOCOL MODIFICATION

For use of this form, see IACUC SOP 1.0

1. DATE: (YYYY/MM/DD) 2011/10/25	2. PROTOCOL NUMBER: 0ENT-24-11-07-04	3. MODIFICATION#: 01

4. PROTOCOL TITLE: Acute Inhalation Toxicity and Blood Absorption of 3-Nitro-1,2,4-Triazol-5-One (NTO) in Rats

5. STUDY DIRECTOR/PRINCIPAL INVESTIGATOR:	6. WORK PHONE:	7. OFFICE SYMBOL:
Arthur J. O'Neill	410-436-5080	MCHB-IP-TTE

SECTION I. PREVIOUSLY APPROVED AND CURRENTLY IN USE PROTOCOL MODIFICATIONS:

1. MODIFICATION NUMBER	2. SHORT DESCRIPTION OF PRIOR APPROVED MODIFICATION(S)	3. NO. & SPECIES OF ANIMAL REQUESTED	4. APPROVED DATE

SECTION II. CHANGE IN TOTAL # OF ANIMALS USED AND/OR CHANGE IN USDA PAIN CATEGORY

1a. CHANGE: INCREASE TOTAL APPROVED ANIMALS BY: 2 ENTRY FROM CORRECTED AO 11/16/11 | 1b. N/A ☒

2. ORIGINAL PROTOCOL TOTAL: 84 ~~30~~ 28 | 3. PROTOCOL TOTAL AFTER MODIFICATION: 86

2a. USDA pain cat:	B: 2	C: 42	D: 12	E: ~~30~~	3a. USDA pain cat:	B: 2	C: 42	D: 12	E: 30

4. Yes	No	
☒	☐	Modification requires specific changes or additions to the experimental design of the protocol. (Section V.I. of the template.)
☐	☒	Modification requires changes to the technical methods, i.e., procedures, routes of administration, biosample collection, etc. (Section V.4. of the protocol template.) Indicate training of personnel for new methods, procedures being used.
☐	☒	Modification requires additions or changes in personnel performing procedures. (Section VI of the protocol template.) Include training and qualification information and tasks that each individual will be performing. If changing the Study Director/PI, a signed Assurance Statement needs to be submitted with the modifications.

PROTOCOL Page, paragraph, section	**SECTION III. MODIFICATION/JUSTIFICATION** *Explain the modification indicated above in the area below. Indicate any changes to the 3R's (Refinement, Reduction, Replacement) resulting from changes in number of animals*
V.1., p. 3,	1. MODIFICATION: Prior to conducting any of the 3 tests outlined in the current protocol, a pilot study will be conducted with 2 rats (one male and one female). The rats will be exposed nose-only to the test atmosphere for approx 4 hours, however, the exposure period may be terminated at any point in time if the health conditions of the rats are considered to be unacceptable. Rats will be observed during the exposure period and weighed/observed up to 3 days following the exposure. Rats will be euthanized with CO2 following the recovery period.
	1a. JUSTIFICATION/REASON: To facilitate generation of the test atmosphere, the test material will be mixed with water. The resulting mixture of NTO in water is acidic (ph~1). In an attempt to mimic the potential exposure conditions of the test material and minimize variables that may mask the true effects of the test material, buffering the test mixture is not considered an option. It is not clear if the test atmosphere that will be generated by aerosolizing the test mixture will pose an unacceptable health issue for the exposed rats. The results from the pilot study will be used to determine if additional testing with NTO by the existing protocol will be continued.

PROTOCOL Page, paragraph, section	*Explain the modification indicated above in the area below. Indicate any changes to the 3R's (Refinement, Reduction, Replacement) resulting from changes in number of animals used.*
V.3.4., p. 14 V.4.1.1.1.4., p 16	2. MODIFICATION: The minimum number of animals to be used on study increases from 28 to 30 rats. The maximum number of animals to be used on study increased from 84 to 86 rats. The number of rats in pain category E increases from 28 to 30 rats.
	2a. JUSTIFICATION/REASON: Two additional rats are needed to conduct the pilot study. The pilot study rats will be exposed to a higher concentration of test material that may be acidic.
	3. MODIFICATION:
	3a. JUSTIFICATION/REASON:
	4. MODIFICATION:
	4a. JUSTIFICATION/REASON:

Continued on next page YES NO X

SECTION IV. SIGNATURES AND DATES

1. STUDY DIRECTOR: (Printed Name) Arthur J. O'Neill	Arthur J. O'Neill	DATE: (yyyy/mm/dd) 2011/10/25
2. PROGRAM MANAGER:: (Printed Name) Dr. Glenn J. Leach	Glenn J. Leach	DATE: (yyyy/mm/dd) 2011/10/25
3. ATTENDING VETERINARIAN: (Printed Name) Dawn C. Fitzhugh, Maj, VC	Dawn C. Fitzhugh	DATE: (yyyy/mm/dd) 2011/10/25
4. CHPPM SAFETY OFFICER/OCC HEALTH REP: (IF APPLICABLE) N/A		DATE: (yyyy/mm/dd)
5. CHAIR, IACUC: (Printed Name) APPROVED YES X NO Kristin T. Newkirk	Kristin T. Newkirk	DATE: (yyyy/mm/dd) 2011/11/02

USACHPPM PROTOCOL MODIFICATION

For use of this form, see IACUC SOP 1.0

1. DATE: (YYYY/MM/DD) 2011/11/02	2. PROTOCOL NUMBER: 0ENT-24-11-07-04	3. MODIFICATION#: 02

4. PROTOCOL TITLE: Acute Inhalation Toxicity and Blood Absorption of 3-Nitro-1,2,4-Triazol-5-One (NTO) in Rats

5. STUDY DIRECTOR/PRINCIPAL INVESTIGATOR:	6. WORK PHONE:	7. OFFICE SYMBOL:
Arthur J. O'Neill	410-436-5080	MCHB-IP-TTE

SECTION I. PREVIOUSLY APPROVED AND CURRENTLY IN USE PROTOCOL MODIFICATIONS:

1. MODIFICATION NUMBER	2. SHORT DESCRIPTION OF PRIOR APPROVED MODIFICATION(S)	3. NO. & SPECIES OF ANIMAL REQUESTED	4. APPROVED DATE
1	Pilot study conducted with 2 rats to determine if test atmosphere generated in exposure chamber will pose an unacceptable health issue for rats	2	2011/11/02

SECTION II. CHANGE IN TOTAL # OF ANIMALS USED AND/OR CHANGE IN USDA PAIN CATEGORY

1a. CHANGE: INCREASE TOTAL APPROVED ANIMALS BY: 0									1b. N/A ☒
2. ORIGINAL PROTOCOL TOTAL: 86					3. PROTOCOL TOTAL AFTER MODIFICATION: 86				
2a. USDA pain cat:	B: 2	C: 42	D: 12	E: 30	3a. USDA pain cat:	B: 2	C: 42	D: 12	E: 30

4. Yes	No	
☒	☐	Modification requires specific changes or additions to the experimental design of the protocol. (Section V.I. of the template.)
☒	☐	Modification requires changes to the technical methods, i.e., procedures, routes of administration, biosample collection, etc. (Section V.4. of the protocol template.) Indicate training of personnel for new methods, procedures being used.
☐	☒	Modification requires additions or changes in personnel performing procedures. (Section VI of the protocol template.) Include training and qualification information and tasks that each individual will be performing. If changing the Study Director/PI, a signed Assurance Statement needs to be submitted with the modifications.

PROTOCOL Page, paragraph, section	**SECTION III. MODIFICATION/JUSTIFICATION** *Explain the modification indicated above in the area below. Indicate any changes to the 3R's (Refinement, Reduction, Replacement) resulting from changes in number of animals*
p. 6-7, V.1.3 p. 19, V.4.4.1	1. MODIFICATION: Urine samples will be collected from the 12 catheterized rats in Experiment 3 (Multi-Timepoint Blood Absoption Test). The 12 rats in Experiment 3 will be placed in a metabolism cage following their 8-hr bleed timepoint. Rats will remain in the metabolism cage overnight and urine samples will be collected. Rats will have food and water available while they are in the metabolism cage.
	1a. JUSTIFICATION/REASON: The urine samples will be analyzed by LS personnel to determine the concentration of the test material and/or metabolites in the urine of rats exposed to NTO. The purpose of this data is to determine if the urine of individuals working with IMX-101 can be used to monitor their exposure to the test material.

PROTOCOL Page, paragraph, section	*Explain the modification indicated above in the area below. Indicate any changes to the 3R's (Refinement, Reduction, Replacement) resulting from changes in number of animals used.*
p. 4-5, V.1.1	2. MODIFICATION: The number of animals to be exposed during the acute inhalation toxicity test will decrease from 10 rats (5 male and 5 female) to 6 rats (3 male and 3 female).
	2a. JUSTIFICATION/REASON: Due to difficulties with the generation of the test atmosphere, the faceplate on the generation system must be changed. The maximum number of rats for the acute inhalation testing that can be accomodated within the new faceplate is six.
p. 14-15, V.3.4	3. MODIFICATION: The minimum number of animals to be used on study changes from 28 rats to 24 rats. The maximum number of animals to be used on study changes from 86 to 64.
	3a. JUSTIFICATION/REASON: The number of rats for the acute inhalation test changes from 10 rats per exposure to 6 rats per exposure.
p. 16, V.4.1	4. MODIFICATION: The following changes are made to the number of rats in the 4 pain categories: (1) Column B remains at 2 rats, (2) Column C decreases to 30 rats, (3) Column D remains at 12 rats, (4) Column E decreases to 20 rats
	4a. JUSTIFICATION/REASON: The number of rats for the acute inhalation test changes from 10 rats per exposure to 6 rats per exposure. For column C, estimation for acute inhalation test is based on 6 rats per exposure at the 3 lower concentrations. For column E, estimation for acute inhalation test is based 6 rats per exposure at the 2 higher concentrations.

Continued on next page YES ☐ NO ☒

SECTION IV. SIGNATURES AND DATES

1. STUDY DIRECTOR: (Printed Name) Arthur J. O'Neill		DATE: (yyyy/mm/dd) 2011/11/15
2. PROGRAM MANAGER:: (Printed Name) Dr. Glenn J. Leach		DATE: (yyyy/mm/dd) 2011/11/16
3. ATTENDING VETERINARIAN: (Printed Name) Dawn C. Fitzhugh, Maj, VC		DATE: (yyyy/mm/dd) 2011/11/15
4. CHPPM SAFETY OFFICER/OCC HEALTH REP: (IF APPLICABLE)		DATE: (yyyy/mm/dd)
5. CHAIR, IACUC: (Printed Name) APPROVED YES ☒ NO ☐ Kristin T. Newkirk		DATE: (yyyy/mm/dd) 2011/11/16